UNIVERSITY OF OSLO

FACULTY OF MEDICINE

Immunopathogenic mechanisms of Coronary Artery Disease and plaque destabilization

Camilla Smith

Research Institute for Internal Medicine

Rikshospitalet-Radiumhospitalet Medical Center

Faculty of Medicine

University of Oslo

Oslo 2007

The Norwegian Council on Cardiovascular Diseases

Series of dissertations submitted to the
Faculty of Medicine, University of Oslo
No. 599

ISBN 978-82-8072-725-1

Cover: Inger Sandved Anfinsen.
Printed in Norway: AiT e-dit AS, Oslo, 2008.

Produced in co-operation with Unipub AS.
The thesis is produced by Unipub AS merely in connection with the
thesis defence. Kindly direct all inquiries regarding the thesis to the copyright
holder or the unit which grants the doctorate.

Unipub AS is owned by
The University Foundation for Student Life (SiO)

Table of contents

Table of contents **3**
1. Acknowledgements **5**
2. Abbreviations **7**
3. List of papers **9**
4. Introduction **10**
4.1 Coronary artery disease – general aspects 10
4.2 Atherogenesis – general aspects 11
4.3 Acute coronary syndromes – shifting focus from stenotic vessels to plaque disruption 12
4.4 The role of inflammation in atherogenesis 13
4.5 Inflammation – potential mediator of plaque destabilization 15
4.6 Chemokines - important players in inflammation 16
4.6.1 Homeostatic and inflammatory chemokines 17
4.6.2 Membrane bound chemokines 18
4.7 The role of chemokines in atherogenesis 21
4.8 Platelets – important cellular actors in atherogenesis 22
4.9 Platelet-mediated inflammation 24
4.10 Anti-inflammatory cytokines 26
4.11 TGF-β superfamily - pleitropic mediators with anti-inflammatory potential 27
5. Purpose of the study **30**
6. Summary of results **31**
Paper I: 31
Paper II: 31
Paper III. 32
Paper IV. 33
Paper V 33
7. Discussion **35**
7.1 Methodological considerations 35
7.1.1 Individuals 35
7.1.2 Blood sampling and chemokine measuring in blood and cell culture supernatants 35
7.1.3 Enzyme Linked Immunosorbent Assays (ELISAs) 36
7.1.4 Analysis of gene expression 36
7.1.5 Cell isolation and culture 37
7.2 Chemokines and atherogenesis 38
7.2.1 NAP-2: another CXCR2 ligand that is involved in atherogenesis and plaque

destabilization 38
7.2.2 CXCL16: a transmembrane chemokine with pro-atherogenic properties 40
7.2.3 CCL19 and CCL21: Inflammatory effects of homeostatic chemokines in CAD 44
7.3 LIGHT: a “new” platelet-derived mediator of inflammation 48
7.4 Activin A: a potential anti-imflammatory actor in CAD and plaque destabilization 51
8. Concluding remarks 54
9. References 56

1. Acknowledgements

This thesis is based on studies performed at the Research Institue for Internal Medicine, Rikshospitalet University Hospital, University of Oslo, during the years 2002-2007. In this period I was a recipient of a research fellowship from the Norwegian Council on Cardiovascular Diseases for which I am very grateful. Other financial support was provided by Medinnova AS.

First and foremost I wish to express my profound and sincere gratitude to my supervisor Pål Aukrust, in whom I am deeply indebted. I am grateful to have had the possibility to share his never ending enthusiasm as well as his enormous amounts of knowledge in all aspects of theoretical and clinical immunology. The preparation of manuscripts under his constructive supervision has been a true pleasure. His good mood, friendly personality, as well everlasting optimism in both successful and not so successful periods is enviable. It is a privilege to have had him as my mentor.

Further, I am deeply grateful to my excellent co-supervisor Jan Kristian Damås. His impressive scientific knowledge along with his ever so positive and enthusiastic personality has been invaluable.

I further want to thank my co-supervisor Knut Endresen for facilitating blood and tissue sampling at the Departement of Cardiology, Rikshospitalet. I also want to thank Prof. Stig Frøland, Section of Clinical Immunology and Infectious Diseases, for contributing with his great knowledge and critical approach to research. Thanks also go to Prof. Frank Brosstad and Prof. Nils Olav Solum for providing me with great working facilities at the Research Institute for Internal Medicine. Thanks are also due to Prof. Lars Gullestad and Prof. John Kjekshus, Departement of Cardiology, for support and sharing their expertise in clinical cardiology.

Further I want to thank my colleague Bjørn Brandsæter for sharing ups and downs in every day life at work, Bente Halvorsen for being inspiring and enthusiastic to any new scientific project, as well as being a positive and warm person, and my room mate Wiggo Sandberg for his unique ability to always participate with great passion in every discussion concerning thinkable and unthinkable subjects. I also want to express my gratitude to all former and present research fellows and colleagues at the Institute; Christen Dahl, Linn Landrø, Erik Øie, Unni Breland, Tuva Dahl, Ellen Lund Sagen, Arne Yndestad, Thor Ueland, Ernst Kristian Rødland, Vigdis Bjerkeli, Børre Fevang, Kari Otterdal, Are Holm, Lars Heggelund, Cybele Cristo, Aina Hognestad, Torgun Wæhre, Satish Aurora, Stine Fougner, Turid Pedersen, Azita Rashidi, Marit Nenseter, Kirsten Holven, Bodil Lunden, Jorunn Bratlie, Hanne Scholtz, Annika Michelsen, Stine Bjørnsen and Maureen Raw.

Thanks also go to our collaboration partners in Sweden represented by Prof. Gøran Hansson, Center for Molecular Medicine, Karolinska Institute, Sweden, for sharing their expertise.

I also want to thank all the patients who unselfishly participated in the studies, without their contribution this work truly would have been impossible.

My warmest thoughts go to my family and friends for encouragement, support and being there for me. Above all, I am deeply grateful to Hans Olav for his ever so positive attitude, warmth, care

and support. Finally, my beloved children Selma, Liv and Martinus-you are everything to me.

2. Abbreviations

- β –TG β-thromboglobulin
- ACS Acute coronary syndromes
- AP1 Activator protein 1
- APC Antigen presenting cell
- ATII Angiotensin II
- BMP Bone morphological protein
- CAD Coronary Artery Disease
- CD40L CD40 ligand=CD154
- CTAPIII Connective tissue-activating peptide III
- DARC Duffy antigen receptor for chemokines
- DC Dendritic cell
- FSH Follicle-stimulating hormone
- GRO Growth Related Oncogene
- HVEM Herpes virus entry mediator
- ICAM-1 Intercellular adhesion molecule-1
- LDL Low Density Lipoprotein
- IFN-γ Interferon-gamma
- LIGHT Homologous to **L**ymphotoxins, exhibits **I**nducible expression, and competes with herpes simplex virus (HSV) **G**lycoprotein for **H**erpes virus entry mediator (HVEM/TR2), a receptor expressed by **T** lymphytes, = TNFSF14
- IL Interleukin
- LOX-1 Lecitin-like oxidized low-density lipoprotein receptor-1
- LPS Lipopolysaccaride
- LTβ Lymphotoxin β
- MCP-1 Monocyte chemoattractant protein-1
- MI Myocardial infarction

- MIP Macrophage inflammatory protein
- MP Microparticles
- MMP Metalloproteinase
- NAP-2 Neutrophil-activating peptide-2
- (NF)κB Nuclear factor- κB
- NO Nitric-oxide
- NSTEMI Non-ST-elevation MI
- oxLDL Oxidized low-density protein
- PBMC Peripheral blood mononuclear cells
- PF-4 Platelet factor-4
- R Receptor
- RANTES **R**egulated upon **A**ctivation **N**ormal **T** cell **E**xpressed and **S**ecreted
- RNA Ribonucleic acid
- ROS Reactive oxygen species
- SMC Smooth muscle cells
- SRA Scavenger receptor A
- SR-PSOX Scavenger receptor that binds phosphatidylserine and oxidized lipoprotein
- STEMI ST-elevation M
- TF Tissue factor
- TGF- β Transforming growth factor-beta
- TNF- α Tumor necrosis factor-alpha
- VCAM-1 Vascular cell-adhesion molecule 1
- VLA-4 Very late antigen 4
- vWF von Willebrand factor

3. List of papers

This thesis is based on the following publications, referred to by their Roman numerals:

I. Smith C, Yndestad A, Halvorsen B, Ueland T, Wæhre T, Otterdal K, Scholz H, Endresen K, Gullestad L, Frøland SS, Damås JK, Aukrust P. Potential anti-inflammatory role of activin A in acute coronary syndromes. J Am Coll Cardiol 2004;44:369-75.

II. Otterdal K, Smith C, Øie E, Pedersen TM, Yndestad A, Stang E, Endresen K, Solum NO, Aukrust P, Damås JK. Platelet-derived LIGHT induces inflammatory responses in endothelial cells and monocytes. Blood 2006;108:928-35.

III. Smith C, Damås JK, Otterdal K, Øie E, Sandberg WJ, Yndestad A, Wæhre T, Scholz H, Endresen K, Olofsson PS, Halvorsen B, Gullestad L, Frøland SS, Hansson GK, Aukrust P. Increased levels of Neutrophil-activating peptide-2 in acute coronary syndromes. Possible role of platelet-mediated vascular inflammation. J Am Coll Cardiol 2006;48:1591-9.

IV. Damås JK, Smith C, Øie E, Fevang B, Halvorsen B, Wæhre T, Boullier A, Breland U, Yndestad A, Ovchinnikova O, Robertson AK, Sandberg WJ, Kjekshus J, Taskén K, Frøland SS, Gullestad L, Hansson GK, Quehenberger O, Aukrust P. Enhanced expression of the homeostatic chemokines CCL19 and CCL21 in clinical and experimental atherosclerosis: possible pathogenic role in plaque destabilization. Arterioscler Thromb Vasc Biol 2007:27:614-20.

V. Smith C, Halvorsen B, Otterdal K, Wæhre T, Yndestad A, Fevang B, Sandberg WJ, Breland U, Frøland SS, Øie E, Gullestad L, Damås JK, Aukrust P. Increased levels and inflammatory effects of soluble CXCL16 in coronary artery disease - down-regulatory effects of statins. Submitted.

4. Introduction

4.1 Coronary artery disease – general aspects

Cardiovascular disease is the most common cause of death in western countries despite changes in lifestyle and the use of new therapeutic modalities to lower systemic cholesterol levels. Atherosclerotic disorders are also recognized as an increasing cause of significant mortality and morbidity in the developing world. A better understanding of the pathogenic mechanisms of this disorder, potentially leading to new treatment modalities, could therefore be of major importance to society.

Atherosclerosis is a progressive disease in large- and medium-sized arteries in which lipids, extracellular matrix and activated smooth muscle cells (SMC) accumulate in the arterial wall resulting in growth of an atherosclerotic plaque.[1, 2] With the formation of fatty streaks, we are already early in life predisposed to this disorder. Fatty streaks, containing mostly lipid-laden macrophages and T-cells, present as asymmetrical focal thickening of the intima. These early plaques though not clinically significant, can later disappear or evolve into more fibrotic and complex lesions characterized by a necrotic core with lipid rich debris surrounded by a cap of SMC and collagen rich matrix, eventually leading to clinical manifestations of coronary heart disease and other forms of atherosclerotic disorders such as cerebrovascular and peripheral artery disease.[1, 2]

With progression of coronary atherosclerosis, the plaque extends eccentrically without compromising the lumen. As the atherosclerotic disease worsens, a luminal encroachment of the plaque can result in hemodynamic obstruction and subsequently symptoms of stable angina pectoris.[1, 2] In contrast to progressive stenosis, acute coronary syndromes (ACS), i.e., unstable angina and acute myocardial infarction (MI), including both ST-elevation MI (STEMI and non-ST-elevation MI (NSTEMI), seem to be caused by a sudden physical disruption of atherosclerotic plaques triggering thrombus formation and vascular obstruction.[3] Two major types of physical disruption of atherosclerotic plaques may occur.[4] Firstly, superficial erosion of the endothelial monolayer uncover subendothelial collagen and von Willebrand factor (vWF) promoting platelet adhesion and activation with subsequent thrombus formation. The second and most common mechanism of plaque disruption involves rupture of the fibrous cap.[4, 5] This cap normally serves to

sequester the thrombogenic lipid-rich core in the atheroma. However, during fissure formation, often occurring in the plaque shoulder, there is a marked activation of the coagulation cascade and platelets, and such mechanisms may account for approximately three-quarter of ACS. However, although some general aspects have been established, the pathogenic mechanisms that lead to plaque destabilization are still incompletely understood.

4.2 Atherogenesis – general aspects

The earliest changes that precede the atherosclerotic lesions take place in the intima which is the innermost layer of an artery, consisting of loose connective tissue and covered by a monolayer of endothelium. The endothelium is a selectively permeable barrier, and at typical predilection sites with decreased shear stress and increased turbulence, alterations in endothelial morphology, permeability as well as surface molecules are seen. At these sites, low-density lipoprotein (LDL) diffuses from the blood into the subendothelial matrix where it can be modified by oxidation, glycosylation, aggregation or associated with proteoglycans and thus trapped in the subendothelial space. The infiltration and retention of LDL in the arterial intima initiate an inflammatory response in the artery wall. Modification of LDL, through oxidation or enzymatic attack in the intima, leads to the release of phospholipids that can activate endothelial cells, preferentially at sites of hemodynamic strain. Patterns of hemodynamic flow typical for atherosclerosis-prone segments (low average shear but high oscillatory shear stress) cause increased expression of adhesion molecules and inflammatory genes by endothelial cells. Therefore, hemodynamic strain and the accumulation of lipids may initiate an inflammatory process in the artery. Thus, although several factors may be involved, the interaction between modified LDL and endothelial cells seem to be the initial event in atherogenesis contributing to several co-existing processes (e.g., inflammation and pro-coagulation) eventually leading to a manifest atherosclerotic plaque.

The degree in witch LDL is modified varies greatly. Through progressive modification by reactive oxygen species (ROS) from macrophages and endothelial cells as well as myeloperoxidases and lipases from neutrophils, it can, as highly oxidized LDL (oxLDL), be internalized in macrophages. This interaction between oxidized modified LDL and macrophages within the atherosclerotic lesion, mediated through various scavenger receptors like CD36, scavenger receptor A (SRA), and Lectin-like oxidized low-density lipoprotein receptor-1 (LOX-1), is a major event in atherogenesis turning macrophages into lipid-laden foam cells. These foam cell

macrophages are important actors in atherogensis within the atherosclerotic lesion, contributing to lipid accumulation, inflammation, matrix degrading and thrombus formation.[2, 6-8]

The formation of a fibrous cap is an important step in atherogenesis. This formation develops as a result of a multi-etiological process. Firstly, death of foam cells through apoptosis or necrosis results in accumulation of extracellular lipids and cell debris. Second, the mutual activation of T cells and macrophages results in production of various cytokines and growth factors witch stimulate SMC proliferation, migration as well as their production of extracellular matrix. Third, a similar influence is seen as a result of elevated homocysteine and blood pressure, clinical findings commonly seen patients with CAD. Increased homocysteine levels may injure the endothelium, at least partly involving ROS-related mechanisms, resulting in stimulation of SMC. As for hypertension, elevated Angiotensin II (ATII) levels may play a pathogenic role. Not only does ATII affect proliferation and hypertrophy of SMC by augmenting intracellular calcium through binding to SMC receptors, it also acts as a potent vasoconstrictor by itself or by decreasing nitric oxide (NO) levels, thus impairing the arterial flow. Hence, with the influx and proliferation of SMC, an elastic fibrous cap is formed, preventing the contact between the blood and the pro-thrombotic material in the lesions. Although this process may lead to narrowing of the arterial lumen, it will also promote plaque stability preventing plaque rupture and development of ACS.[2, 7, 9-15]

4.3 Acute coronary syndromes – shifting focus from stenotic vessels to plaque disruption

Our classical view held that acute MI usually occurred due to a critically narrowed coronary arterial lumen, detectable by angiography. However, careful pathologic and angiographic studies in the 1980s determined that fissuring or rupture of the thin fibrous cap of a coronary atheroma with preserved lumen often triggers acute fatal thrombosis. Thus, angiographic studies have shown that the culprit lesion in acute MI may not necessarily cause hemodynamically relevant (flow-limiting) stenosis of the coronary arteries.[16] Moreover, several large clinical studies have shown that while cholesterol lowering with statins substantially reduces the occurrence of acute adverse coronary events, such therapy surprisingly produces only slight reduction in arterial obstruction estimated by angiography.[17, 18] A striking difference between patients with unstable and stable angina is the higher incidents of new coronary events in the unstable group.[19] Surprisingly, in

almost half of the cases such recurrent events are unrelated to the initial culprit lesion but arise from complications in other segments of the coronary vasculature. In support of this notion, several angiographic and angioscopic studies have revealed that all major coronary arteries are widely diseased displaying multiple vulnerable plaques in unstable patients.[20, 21] These new findings have shifted the goal of therapy towards plaque stabilization rather than enlargement of the lumen. In order to achieve such a goal, a better understanding of the biology of the atherosclerotic plaques and the processes leading to plaque destabilization is needed.

4.4 The role of inflammation in atherogenesis

Atherosclerosis is a progressive multifactor disease in which lipids, extracellular matrix, and activated vascular SMC accumulate in the arterial wall resulting in growth of an atherosclerotic plaque. Recent research has shown that inflammation plays a key role in this process. Hence, immune cells dominate early atherosclerotic lesions, their effectors molecules accelerate progression of the lesions, and activation of inflammation can elicit ACS.[7, 14, 22]

In this inflammatory disorder, leukocyte recruitment and transmigration to the vascular lesions, which is regulated through a multistep process where functions mutually overlap, is a major pathogenic event. The initial adhesion or rolling of leucocytes to the endothelium is mediated by selectins such as intercellular adhesion molecule-1 (ICAM-1), platelet selectin (P-selectin) and endothelial selectin (E-selectin), and their binding to carbohydrate ligands on the leucocytes. Further, through an integrin-mediated arrest on the activated endothelium, a firm adhesion is made when monocytes and T cells bind to vascular cell-adhesion molecule-1 (VCAM-1) expressing endothelial cells through their intergrin very late antigen-4 (VLA-4). A subsequent transmigration or transendothelial diapedesis of leukocytes to subendothelial tissue is induced by and towards a locally produced chemokine gradient generated of, among others, monocyte chemoattractant protein-1 (MCP-1), interleukin (IL)-8 and Regulated upon Activation Normal T cell Expressed and Secreted (RANTES). Cytokines such as chemokines, tumor necrosis factor-alpha (TNF-α), IL-1 and interferon-gamma (IFN-γ) play a major role in this process leading to endothelial cell activation and recruitment and activation of leukocytes into the atherosclerotic lesion. Leukocyte infiltration and activation within the atherosclerotic plaque will further lead to increased production of inflammatory cytokines which again will further enhance leukocyte infiltration, as part of a pathogenic loop in atherogenesis. This persistent inflammation will also alter the

phenotype of macrophages and SMC within the atherosclerotic lesion. Thus, monocytes differentiate into macrophages in response to, among others, local production of macrophage colony stimulating factor (M-CSF). A further development to foam cells through uptake of modified lipoproteins by scavenger receptors is vastly regulated by cytokines such as TNF-α, IL-6 and IFN-γ, turning macrophages into lipid-laden foam cells with an enhanced inflammatory, pro-coagulant and matrix degrading potential. Moreover, inflammatory cytokines may also transform SMC from a contractile to a proliferative/secretory phenotype which is a hallmark of the vascular remodeling characterizing atherogenesis. [2, 7, 8, 12, 23]

The presence of activated T cells in all stages of atherogenesis implies that also these cells are involved in aggravation of disease. Indeed, T cell deficient severe combined immunodeficiency mice on an apoE-knockout background develop less atherosclerotic disease than do immunocompetent apoE-knockout mice.[24] On the other hand, transfer of $CD4^+$ T cells into these immunodeficient atherosclerotic miceaccelerates the disease.[24] However, while the pathogenic role of T cells in atherogenesis is well established, the complex function of the various T cell subsets in this disorder is far from clear. This, while activation of the so-called regulatory T cells, producing IL-10 and transforming growth factor-beta (TGF-β), may be an interesting antiatherogenic target, activation of the inflammatory T helper type 1 (Th1) subset, leading to increased production of IFN-γ, IL-2 and TNF-α, seems to be major atherogenic event promoting activation of macrophages and vascular SMC within the lesion.[25] However, although the participation of inflammation in atherogensis is well established, the characterization of the various actors as well as their regulation and relative importance is far from clear.

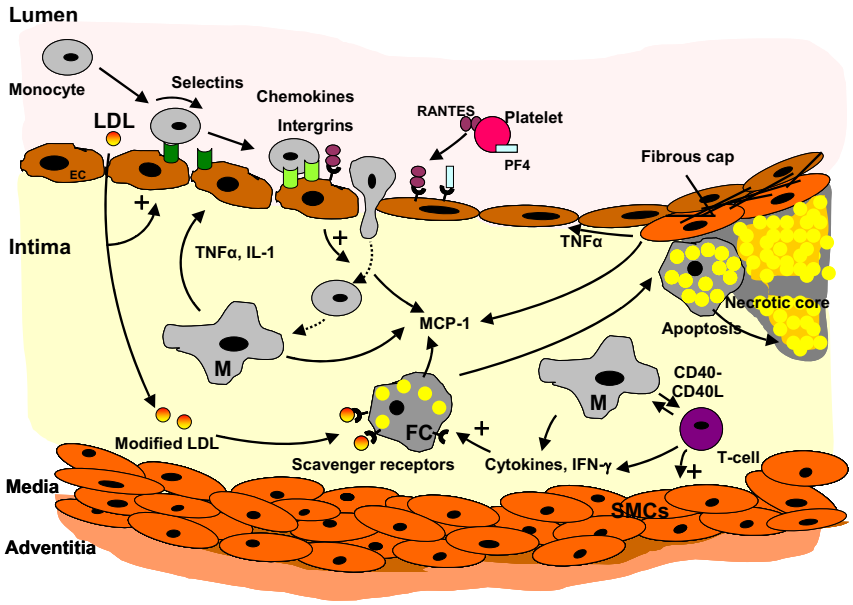

Figure 1. Cytokines and chemokines in mononuclear cell recruitement and development of atherosclerotic lesions. A triggering event for leucocyte transmigration to the vessel wall is the accumulation of intimal modified lipoprotein particles with a subsequent stimulation of the overlying endothelial cells to produce adhesions molecules such as selectins and integrins, as well as chemotactic proteins such as MCP-1. According to the multistep paradigm, leucocyte trafficking is initiated by selectin-mediated rolling followed by an integrin-dependent arrest on cytokine activated endothelium and transmigration induced by chemokines. Chemokine (i.e., RANTES and PF4) deposition on endothelial cells from activated platelets also contribute to the arrest of leucocytes. Extravasated macrophages (M) recognize LDL by scavenger receptors (i.e., SR-A and CD36) whose expression is mediated by cytokines such as TNF-α and INF-γ. Through accumulation of modified LDL they transform into foam cells (FC). The death of foam cells results in a growing mass of extracellular lipids and debris, a necrotic core. The interaction of CD40 and CD40L stimulates T-cells and macrophages to express cytokines that can influence inflammation, SMC proliferation and migration with subsequent SMC derived extracellular matrix giving rise to a fibrous cap.

4.5 Inflammation – potential mediator of plaque destabilization

Several lines of evidence suggest that inflammation is an integral part not only of the chronic atherosclerotic process, but also of plaque destabilization leading to the development of ACS. Thus, elevated circulating levels of inflammatory mediators such as inflammatory cytokines are found in patients with angina, particularly in those with unstable disease.[26] Also, activated monocytes, T-cells and granulocytes have been demonstrated in patients with unstable angina with

particularly enhanced activation in cells isolated from coronary sinus.[27] Moreover, accumulation of inflammatory cells such as macrophages and T-cells seems to be an important feature of the active stages of atherosclerosis.[14] Hence, in atherectomy specimens and necropsy studies, disrupted plaques typically display a large lipid core, thin fibrous cap, reduced number of smooth muscle cells accompanied by a large numbers of monocyte-derived macrophages and activated T-cells.[28-30] Furthermore, it seems that the pan-coronary vulnerability during ACS may result from a widespread coronary inflammation. Hence, Buffon *et al* have recently reported a transcoronary neutrophil activation in patients with unstable angina, occurring to a similar degree in the presence or absence of the culprit lesion.[31] Moreover, inflammatory cytokines could promote tissue factor (TF) expression in macrophages, change endothelial cells into to a pro-coagulant phenotype by up-regulate plasminogen activator inhibitor-1 (PAI-1) and down-regulate thrombomodulin expression as well as induce matrix metalloproteinase (MMP) activity in SMC and macrophages, promoting thrombus formation and matrix degrading within the atherosclerotic lesion.[32] Finally, multiple studies have established inflammatory markers and in particular C-reactive protein (CRP) as markers of risk of future cardiovascular events.[33, 34] Thus, CRP has proven remarkably robust as a marker of cardiovascular risk and has been found to give predictive value beyond that of traditional risk factors in CAD patients.

Thus, it seems that complex and not yet clarified immunological mechanisms regulate not only the initiation but also the outcome of the atherosclerotic process, emphasizing the importance of further studies of these inflammatory mechanisms, trying to identify new targets for therapy in this disorder.

4.6 Chemokines - important players in inflammation

Chemokines, *chem*otactic cyto*kines*, belong to a superfamily of chemoattractant cytokines involved in leukocyte recruitment and activation. These small polypeptide molecules of 8-10 kDa can be induced by cytokines, growth factors and some pathogenic stimuli such as oxidative stress and various microbes. The approximately 50 known chemokines can be segregated into four families on the basis of the number and spacing of the conserved cysteine residues in their sequences; — C–C, C–X–C, C–X_3–C and C — (Table1). The families of CXC, having a single amino acid interposed (e.g., IL-8 and growth related oncogene [GRO]-α), and CC (e.g., MCP-1 and RANTES) constitute the two major families. The third family, CX3C, contains fractalkine

(CX3CL1) as the only member, and the fourth C family comprises lyphotacins.[35, 36] The CXC family can be further subdivided into two categories depending on the presence or absence of a three amino acid sequence, a glutamate-leucine-arginine (ELR) motif. In general the ELR^+ chemokines are involved in the early inflammatory phase through attracting neutrophils, while ELR^- chemokines act on lymphocytes. Moreover, the two different ELR group also have different aniogenic properties. Thus, while ELR^+ chemokines are potent angiogenic factors, the ELR^- chemokines are anti-angiogenic. CC chemokines act primarily on monocytes, eosinophils, basophils and lymphocytes, but in the general there are more overlapping functions between CXC and CC chemokines than previously recognized.[37]

Each family of chemokines has a reciprocal family of seven-transmembrane G-protein-coupled receptors, whereas most receptors bind more than one chemokine. Receptor activation can result in an array of events. Changes in cell shape can occur within seconds, facilitating cell movement toward the chemokine gradient. Also intracellular events are affected through changes in gene expression. Further, receptor activation can promote the release of contents in cytoplasmatic granules such as proteases, and induce $Ca2^+$ influx to the cell with a subsequent generation of oxygen radicals and lipid mediators. Interestingly, chemokine receptors are constitutively expressed on some cells whereas inducible in others. Also, some constitutive receptors can be down-regulated, while the expression of others is restricted to a cell state of activation and differentiation. The chemokine receptor phenotype of inflammatory cells fluctuates during their differentiation and exposure to external stimuli. Duffy antigen receptor for chemokines (DARC) interacts with non-signaling molecules and act as a sink for chemokines, clearing them from the circulation. It is also the only known chemokine receptor that can bind both CC and CXC chemokines. The rapid binding of IL-8 to DARC may at least partly explain the low levels of IL-8 and perhaps also some other chemokines in the circulation.[38-41]

4.6.1 Homeostatic and inflammatory chemokines

Chemokines were originally discovered through their ability to recruit various cell types into sites of inflammation, but it is now clear that they posses a wider role in immune homeostasis driving maturation, homing and activation of leucocytes. In this aspect, one can make a division into two functional classes of chemokines; the inflammatory and the homeostatic chemokines.

Homeostatic chemokines are involved in the regulation of physiological lymphocyte

trafficking, a sort of a housekeeping function, as well as a directional migration and positioning within secondary lymphoid organs and tissues. There is a restricted constitutive production of the homeostatic chemokines (e.g., CC ligand [CCL]19, CCL21 and CXCL13), and elevated levels are found in thymus, lymph nodes as well as other lymphoid tissue interacting with their corresponding chemokine receptors, CCR7 and CXCR5, respectively. However, while the role of these chemokines in the migration of lymphocytes home from their sites of lymphopoiesis to secondary lymphoid organs where they encounter antigen in specialized compartments, or they egress from lymph nodes and eventually recirculate, recent studies suggest that these homeostatic chemokines also may regulate the migration of T and B lymphocytes through non-lymphoid tissues.[38, 42-44]

In contrast to the homeostatic chemokines that are constitutively expressed within secondary lymphoid organs, inflammatory chemokines are up-regulated during various inflammatory events. Inflammatory chemokines promote leukocyte infiltration and activation at the site of inflammation and their expression is inducible, primarily by inflammatory cytokines such as IL-1, TNF-α and IL-12 as well as by enhanced oxidative stress and toll-like receptor (TLR) activation (e.g., TLR2 and TLR4), involving activation of transcriptional factors such as nuclear factor κB (NFκB) TNF-α). Importantly, also traditional cardiovascular risk factors such as high levels of oxLDL and smoking (through ROS generation) might through monocyte activation or direct effects on T cells, lead to increased production of inflammatory chemokines.[2, 11, 32, 45, 46]

The inflammatory CC chemokine MCP-1 is the most thoroughly characterized chemokine, and a key mediator in monocyte trafficking, recruiting them to sites of trauma, bacterial infection and ischemia. The CXC chemokine IL-8 is another prototypic inflammatory chemokine, and similar to MCP-1, IL-8 will not only induce chemotaxis through recruiting inflammatory cells (for IL-8, principally neutrophils, the signature cell of acute inflammatory response), but also stimulate them to a higher activation state. Being rapidly synthesized at local sites of inflammation, resistant to relatively harsh external conditions persisting for a prolonged time, IL-8 is an ideal molecule to live at site of inflammation. [23, 47]

4.6.2 Membrane bound chemokines

Within the chemokine family of small chemotactic polypeptides CX3CL1 and CXCL16 are exceptional in that they are synthesized as transmembrane molecules and can be cleaved from the

cell surface to produce a soluble chemoattractant. As transmembrane molecules on the surface of endothelial cells, CX3CL1 and CXCL16 can interact with their receptors CX3CR1 and CXCR6, respectively, which are expressed on leukocyte subtypes. This interaction leads to cell-cell adhesion that is resistant to shear forces. Functionally, both chemokines appear to exert homeostatic and inflammatory activities. Thus, while basal expression of CX3CL1 or CXCL16 may be relevant for positioning and survival of tissue-homing leukocytes, increased expression have been found during various inflammatory condition potentially contributing to leukocyte recruitment into inflamed tissue. Interestingly, it seems that the membrane-bound and the soluble form of these chemokines may exert different properties, but it remains to be clarified if some of the forms have predominantly a homeostatic or inflammatory function. In fact, the exact role of these distinct chemokines is not fully understood, and it is a challenge for future research to dissect and clarify the role of both the transmembrane and soluble forms of these chemokines in the pathophysiology of various human diseases.[48, 49]

Table 1. Chemokines and their receptors

Family	Functual classification	Systematic name	Human ligand	Chemokine receptors
CC	Inflammatory	CCL1	I-309	CCR8
		CCL2	MCP-1	CCR2
		CCL3	MIP-α	CCR1, CCR5
		CCL4	MIP-1	CCR5
		CCL5	RANTES	CCR1, CCR3, CCR5
		CCL6	Unknown	Unknown
		CCL7	MCP-3	CCR1,CCR2,CCR3
		CCL8	MCP-2	CCR3,CCR5c
		CCL9/10	Unknown	CCR1
		CCL11	Eotaxin	CCR3
		CCL12	MCP-5	CCR2
		CCL13	MCP-4	CCR2, CCR3
		CCL14	HCC-1	CCR1, CCR5
		CCL16	HCC-4	CCR1, CCR2
		CCL23	MPIF-1	CCR1
	Inflammatory, angiogenic	CCL15	HCC-2	CCR1, CCR3
	Inflammatory and homeo-static	CCL17	TARC	CCR4
		CCL22	MDC	CCR4
	Homeostatic	CCL18	PARC	Unknown
		CCL19	ELC/MIP-3β	CCR7
		CCL20	LARC/MIP-3α	CCR6
		CCL21	SLC	CCR7
		CCL2	Eotaxin-2	CCR3
		CCL5	TECK	CCR9
		CCL26	Eotaxin-3	CCR-3
		CCL27	CTACK	CCR10
		CCL28	MEC	CCR3, CCR10
C	Inflammatory	XCL1	Lymfotacin	XCR1
		XCL2	SCM-1β	XCR2
CXC	Inflammatory, angiogenic	CXCL1	GRO-α	CXCR2, CXCR1
		CXCL2	GRO- β	CXCR2
		CXCL3	GRO-γ	CXCR2
		CXCL5	ENA-78	CXCR2
		CXCL6	GCP-2	CXCR1, CXCR2
		CXCL7	NAP-2	CXCR1, CXCR2
		CXCL8	IL-8	CXCR1, CXCR2
	Inflammatory, angiostatic	CXCL9	MIG	CXCR3
		CXCL10	IP-10	CXCR3
		CXCL4	PF4	Unknown
		CXCL11	I-TAC	CXCR3a
	Homeostatic, angiogenic	CXCL12	SDF-1	CXCR4
	Homeostatic	CXCL13	BCA-1	CXCR5
		CXCL14	BRAK	Unknown
	Inflammatory	CXCL16		CXCR6
CXXXC	Inflammatory	CX3CL1	Fractalkine	CX3CR1

I-309, a nameless human chemokine; MCP-1, monocyte chemoattractant protein; MIP, macrophage inflammatory protein; RANTES, regulated upon activation normal T cell expressed and secreted; HCC, human CC chemokine; MPIF, myeloid progenitor inhibitory factor; TARC, thymus-and-activation-regulated chemokine; MDC, macrophage-derived chemokine; PARC, pulmonary and activation-regulated chemokine; ELC, (Ebl-1), EBL-1-ligand chemokine, LARC, liver- and activation-regulated chemokine, SLC, secondary lymphoid tissue chemokine, TECK, thymus-expressed chemokine; CTACK, cutaneous T-cell-activating chemokine; MEC, mucosae-associated epithelial chemokine; SCM, Single C motif; GRO, growth related oncogene; ENA, epithelial neutrophil activating; GCP, granulocyte chemotactic protein; NAP, neutrophil-activating peptide; MIG, monokine-induced by IFN-γ; IP, IFN-γ inducible protein; PF, platelet factor; I-TAC, IFN-inducible T-cell a chemoattractant; SDF-1, stromal cell-derived factor 1; BCA, B-cell attracting chemokine; BRAK, breast and kidney-expressed chemokine.

4.7 The role of chemokines in atherogenesis

Several lines of evidence support an important role for chemokines in atherogenesis. Thus, there are several reports of raised serum levels of chemokines in CAD.[50] Moreover, these chemotactic cytokines seem to be raised not only in circulation, but also within the atherosclerotic lesions.[15] Hence, there are several reports of enhanced expression of both CXC-chemokines (e.g IL-8 and interferon-γ-inducible-10 [IP-10]), CC-chemokines (e.g., MCP-1, leukotactin-1 [Lkn-1], and RANTES) as well as some of their corresponding receptors within atherosclerotic lesions. In addition to being a potent chemoattractant, several other leukocyte responses such as cell proliferation, enzyme secretion and induction of ROS, have been observed *in vitro* after chemokine stimulation).[15] Moreover, beyond their effects on leukocytes, chemokines may also interfere with SMC migration and growth, as well as platelet activation.[51, 52] Some of these responses may clearly be relevant to atherogenesis, and indeed, the co-expression of chemokines and their receptor within atherosclerotic lesions, involving various cell types such as T cells, macrophages, and vascular SMC, suggests their involvement not only in the regulation of lymphocyte recruitment into atherosclerotic lesions, but also in other processes with relevance to atherogenesis such as regulation of SMC phenotype. Furthermore, recent in *vivo* studies have shown that targeted disruption of the genes for MCP-1, CCR2 (i.e., MCP-1 receptor), CXCR2 (i.e., IL-8 receptor) and CX3CR1 (i.e., fractalkine receptor) significantly decreases atherosclerotic lesion formation and lipid deposition in mice prone to develop atherosclerotic lesions.[53-55] These and other studies in gene modified mice, strongly suggest an important pathogenic role of chemokines and atherogenesis.

Notably, infiltration and activation of circulating T cells and monocytes into the atherosclerotic plaque may also be involved in the triggering of ACS.[3] Again, chemokines may

play an important role in this immune-mediated plaque destabilization, not only by recruiting activated leukocytes into the atherosclerotic vessel wall, but also by directly contributing to plaque rupture and thrombus formation by enhancing the matrix degrading potential in macrophages by inducing TF and matrix MMPs in vascular SMC, and by promoting neovascularization within the atherosclerotic lesion which in turn may act as a conduit for the entry of leukocytes into sites of chronic inflammation.[56-61] Chemokines could also promote plaque rupture by enhancing oxidative stress and apoptosis within the atherosclerotic lesions. In fact, angina patients have been found to have raised levels of both CC and CXC chemokines with particularly high concentration of IL-8, MCP-1, and macrophage inflammatory protein (MIP)-1α in unstable disease, significantly correlated with enhanced oxidative stress in these patients.[62] Consequently, chemokine receptor/ligand could be identified as potential important pathogenic mediators not only in the chronic atherosclerotic process, but also in plaque destabilization with subsequent development of ACS. However, their exact role as well as their relative importance is still unclear and much remains to be done to further enlighten the complexity of chemokine responses.

4.8 Platelets – important cellular actors in atherogenesis

Platelets are blood cell fragments originating from megacaryocytes. With a mean cell surface of 8 μm these anuclear, discoid cells circulate in the blood in a resting state with a circulating life of 7-10 days. They contain a contractile system consisting of actin and myosin forming a cytoplasmatic three dimensional network as well as a network of shorter actin fibres and bundles of microtubles serving as the membrane skeleton and maintaining the discoid shape of the resting platelet. Platelets contain remnants of magacaryocyte ribonucleic acid (RNA), mitochondria, and three distinct granules; the electron dense granules, α-granules and lysosomes which contain highly potent substances essential for haemostatic and inflammatory processes. Receptors responsible for adhesion, aggregation and signal transduction are located in the plasma membrane.

When platelets are activated, for example through injury to the vascular wall with a subsequent adherence to the subendothelium, a further initiating of a cascade of signals from the membrane to the cytoplasm is seen. Furthermore, platelets undergo morphological changes, a so called outside-in signaling. Platelets go from being discoid with homogenous distribution of granules to spherical with centralized granules and pseudopodia originating from the plasma membrane.[63] This much more efficient platelet undergoes exocytosis of its centralized granules,

not only resulting in an extracellular milieu able to attract more platelets, but through internalizing new proteins like P-selectin and CD40ligand (CD40L) in the plasma membrane, mediate platelet binding to neutrophils, monocytes and endothelial cells.[63-65] Activation of platelets is also associated with the binding of fibrinogen and/or vWF to their major receptor GPIIb-IIIa which is essential for platelet bridging and subsequent platelet aggregation.[66]

Later events in the platelet activation cascade include rearrangement of the platelet membrane converting it to a procoagulant surface.[67] Microparticles (highly procoagulant small vesicles formed from the platelet surface), as well as adhesion molecules, such as soluble P-selectin, are shed from the platelet surface into the circulation as stimuli for leukocytes and endothelial cells. Taken together, these events culminate in the assembly of the prothrombinase complex leading to formation of a fibrin containing platelet plug and subsequent clot retraction.[67-71]

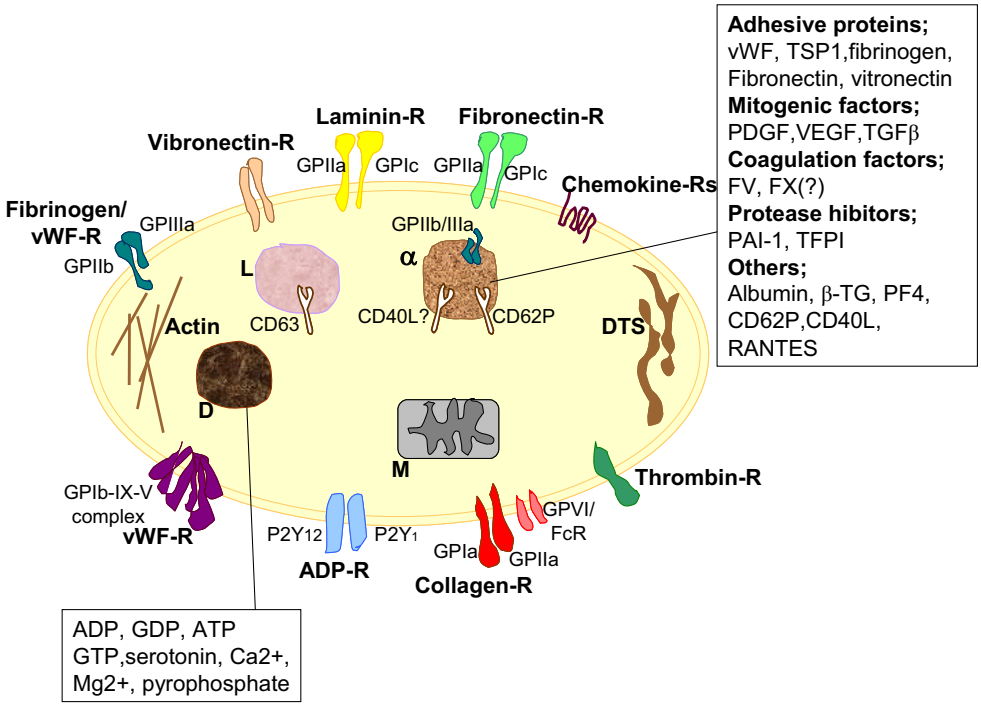

Figure 2. Resting platelet illustrating the different cell compartments and organelles with contents. α, α-granule; β-TG, β-thromboglobulin; ADP, adenosine diphosphate; CD62P, P-selectin; D, dense granules; DTS, dense tubular system; FV, Factor V; GDP, guanosine diphosphate; GP, glycoprotein; L, lysosome; M, mitochondria, PAI-1, plasminogen activator inhibitor-1; PDGF, platelet-derived growth factor; PF4, platelet factor-4; R, receptor; RANTES *R*egulated upon *A*ctication *N*ormal *T* cell *E*xpressed and *S*ecreted; TFPI, tissue factor pathway inhibitor; TGF- β, transforming growth factor-β; TSP, thrombospondin; VEGF, vascular endothelial growth factor; vWF, von Willebrand factor.

4.9 Platelet-mediated inflammation

It is well established that platelets contribute to the pathogenesis of atherosclerosis and ACS by promoting thrombus formation. However, recent studies suggest that these cells also may trigger an acute coronary event through other mechanisms, i.e., stimulation of an inflammatory response within the atherosclerotic plaque. Thus, along with their traditional role in thrombosis and haemostasis, a growing body of evidence links platelets to an array of other processes including inflammatory responses. Firstly, a variety of growth factors and inflammatory mediators are stored

in α-granules and released upon activation. These include platelet factor-4 (PF-4), β-thromboglobulin (β-TG), RANTES, GRO-α along with recent reported CD40L.[64, 72-74] Second, platelets do not only contain and express inflammatory mediators, but may upon activation also induce the expression of such substances in monocytes/macrophages.[75] Actually, upon activation platelets express P-selectin on their surface. Through ligation with its counterpart on monocytes/macrophages, P-selectin has the potential to enhance the activation of the transcriptional factor NFκB[76], a factor required for expression of chemokines, TNF-α and several other gene products playing a key role in inflammation. Notably, recent studies suggest that platelets also may modulate the function of other leukocyte subsets (e.g., natural killer cells, neutrophils, and T cells) and they have been found to enhance chemotactic and adhesive properties of endothelial cells as well as IL-1 production in vascular SMC.[77, 78] Finally, platelets may not only promote an inflammatory response in leucocytes and endothelial cells, but may also themselves respond to inflammatory mediators produced by these cells. In fact, platelets have recently been found to express several chemokine receptors that upon stimulation endorse platelet activation.[79]

Recently, much attention has been focused on the role of platelet-derived CD40L in this inflammatory loop between platelets and other cells. CD40L, a transmembrane protein belonging to the TNF superfamily, was originally identified on $CD4^+$ T cells, but has recently also been found on mast cells, basophils, eosinophils as well as on activated platelets.[80] Soluble CD40L (sCD40L) is found elevated in coronary disease in particular in patients with ACS[81], and has been associated with increased cardiovascular risk in apparently healthy women.[82] Several lines of evidence suggest that these findings not only represent epiphenomena, but also may reflect important pathogenic processes in these patients. Both membrane-bound and sCD40L may interact with CD40, which is constitutively expressed on a wide range of cells such as macrophages, endothelial cells, and vascular smooth muscle cells as well as on platelets, resulting in various inflammatory responses.[80] Thus, *in vitro* stimulation of CD40 signaling in atheroma-derived cells results in the production of cytokines, TF, MMPs and adhesion molecules.[83-85] *In vivo*, an important role for the CD40L-CD40 interaction in atherogenesis was demonstrated using mice deficient in CD40L and apoE showing a dramatic decrease in plaque area in these mice compared with normal apoE-deficient animals.[86] The possible plaque stabilizing effect of CD40L neutralization was further demonstrated in another study where the administration of anti-CD40L antibody to apoE-deficient mice induced a stable plaque phenotype.[87] However, and with

relevance to other cytokines, the relative importance of the soluble and membrane-form of CD40L in mediating its biological effects is still debated. Moreover, recent studies suggest that release mechanism of sCD40L from platelet are different from that of α–granula release[88, 89-91], further complicating the regulation and biological significance of this platelet-derived member of the TNF superfamily. Nevertheless, it is now well established that upon activation platelets may release and express inflammatory mediators, induce an inflammatory response within leucocytes, and respond with activation to several of the inflammatory mediators produced by these cells.[92] This platelet-leucocyte cross talk seems to involve a wide range of mediators such as chemokines, adhesions molecules, ROS, and cytokines.[93-95] It is tempting to hypothesise that this inflammatory interaction between platelets and leucocytes, also involving endothelial cells, may represent a vicious circle playing a pathogenic role not only in the chronic atherosclerotic process, but also in the triggering of ACS.

4.10 Anti-inflammatory cytokines

A wide range of studies have established that CAD patients have increased levels of inflammatory cytokines, but less focus has been drawn to the importance of anti-inflammatory mediators. Among several anti-inflammatory mediators, IL-10 has been paid specially interest, and it has been showed that while unstable angina patients are characterized by markedly elevated plasma levels of inflammatory cytokines, no or only a modest increase in IL-10 levels has been reported in these patients[96, 97], suggesting an inflammatory imbalance. Moreover, low IL-10 level in plasma has been found to be associated with poor clinical prognosis after ACS.[98] IL-10 is a pleiotropic cytokine produced by T-cells, B cells, monocytes and macrophages, and it exhibits a variety of effect with relevance to atherogenesis such potent inhibition of various inflammatory cytokines, induction of anti-inflammatory mediators (e.g., IL-1 receptor antagonist and soluble TNF receptors), inhibition of MMP-9 combined with up-regulation of its endogenous tissue inhibitor (i.e., TIMP-1), down-regulation of TF expression in monocytes as well as anti-apoptotic effects on foam cells suggesting anti-atherogenic and plaque stabilizing effects.[97, 99, 100] Moreover, studies in IL-10 transgenic and IL-10 deficient mice models have suggested an important role of this cytokine in both formation and stabilization of atherosclerotic lesions *in vivo*.[101, 102] In fact, it has been proposed that IL-10 could be regarded as an immunological scalpel within the atherosclerotic vessel wall, being a mediator with therapeutic potential in atherosclerosis. [99, 103]

4.11 TGF-β superfamily - pleitropic mediators with anti-inflammatory potential

TGF-β and related cytokines is another group of mediators with potential anti-inflammatory and anti-atherogenic effects. Thus, Robertson et al. have shown that apoE$^{-/-}$ mice with abrogated TGF-ß signaling in T cells developed dramatically accelerated atherosclerosis with a several-fold increase in lesion size as well as a more vulnerable lesion phenotype with reduced collagen and increased inflammation, further suggesting a protective role for this cytokine in atherogeneis.[104]

TGF-β superfamily is a large family of structurally related polypeptides comprising more than 30 members. This family of growth and differentiation factors contains 3 subgroups; the prototypic TGF-βs, the activins and the bone morphological proteins (BMPs). They control cellular functions like proliferation, differentiation, adhesion and homeostasis by modifying specific sets of genes. The TGF- β superfamily elicits its effect through serine/threonine receptors, classified as type I or II, leading to a subsequent phosphorylation and activation of specific intracellular mediators called Smads. Firstly, the receptor regulated Smads (R-Smads) are presented to the receptor complex by a binding protein called SARA or "Smad anchor for receptor activation" for a subsequent phosphorylation. Once phosphorylated, the R-Smads dissociate from both SARA and the receptor complex. Moreover, phosphorylated R-Smad recruit the co-Smad, Smad4, whereby the complex translocates from the cytoplasm to the nucleus where they bind directly to the DNA and thus control gene expression. These actions are opposed by the inhibitory I-Smads, Smad6 and Smad7, which form complexes with the activated receptors and thus prevent Smad phosphorylation and activation.[105, 106]

The TGF-β family contains unique and pleiotropic proteins exerting an array of functions. They are produced by and act on a wide range of cells, but depending on total cytokine milieu present and state of cell differentiation, they present dichotomous functions. Acting in both an autocrine and paracrine matter, they can both inhibit and stimulate the immunological response.[107-110] Administration of TGF-β is shown to be beneficial in septic shock. In mice models, TGF-β producing cells protect against colitis in inflammatory bowel disease.[111] Moreover, in TGF-β null mice, loss of TGF-β ligand results in progressive tissue inflammation and inflammatory disorders. On the other hand, TGF-β induced deposition of extracellular matrix on site of injury can lead to scarring and fibrosis, the latter associated with chronic inflammatory disorders.[112] Thus, lines of evidence couple TGF-β to the pathogenesis of autoimmune and inflammatory disorders, though in

conflicting roles.[113]

The activins, a subgroup in the TGF-β family, were initially discovered as inducers of follicle-stimulating hormone (FSH), but have shown to hold a wider range of functions concerning cell growth and development. In mice models they are found to play a role in tissue repair and inhibit proliferation of intestinal epithelium in inflammatory bowel disease.[114] There are also some studies implicating activin A in the pathogenesis of rheumatoid arthritis and sepsis possibly mediating anti-inflammatory net effects, but the results are somewhat conflicting. Recent studies suggest that activin A could be involved in atherogenesis by inhibiting foam cell formation and inducing differentiation of neointimal SMC.[115] Given its association with inflammatory disorders, potentially mediating anti-inflammatory effects, it is therefore tempting to hypothesize a potential attenuating role of activin A in atherogenesis and plaque destabilization.

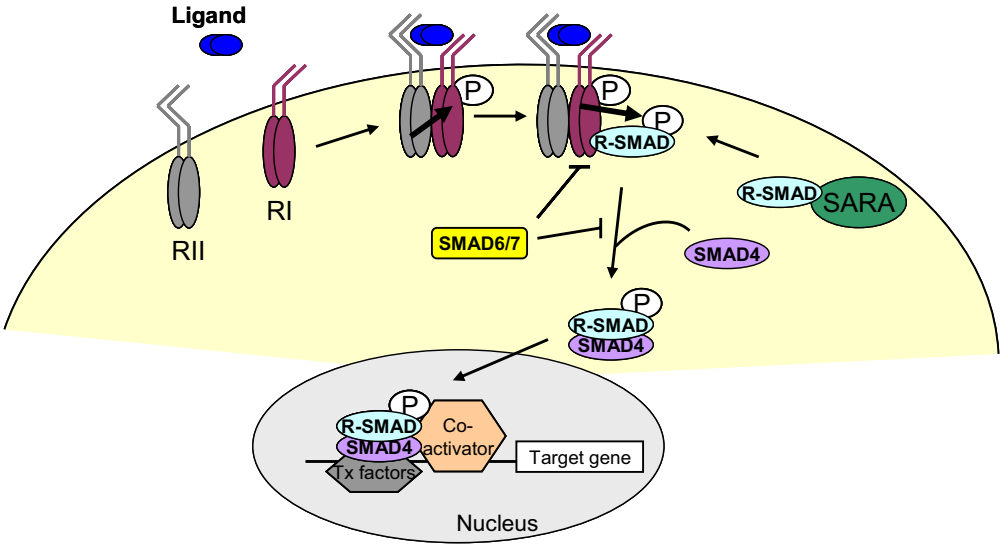

Figure 3. Overview of signal transduction in the TGF-β superfamily.

Binding of ligands leads to the formation of a complex of receptor serine/threonine kinases (type I and II) and transphosphorylation of the type I receptor by the type II receptor. The activated type I receptors phophorylate receptor regulated Smads (R-Smads) which are presented to the receptor complex by a binding protein called SARA or "Smad anchor for receptor activation". Once phosphorylated, the R-Smads dissociate from both SARA and the receptor complex. Moreover, phosphorylated R-Smad recruit the co-Smad Smad4, whereby the complex translocates from the cytoplasm to the nucleus where they bind directly to the DNA and regulate transcription through interaction and cooperation with transcription (Tx) factors and co-activators. Both the phosphorylation of R-Smads and the interaction between R-Smads and Smad 4 is inhibited by the inhibitory Smads, Smad6 or Smad7.

5. Purpose of the study

In this thesis we attempted to further investigate the immunopathogenic mechanisms in CAD and plaque destabilization, particularly focusing on:

a. The pathogenic role of chemokines.
b. The pathogenic role platelet-mediated inflammation.
c. The pathogenic role of activin A as a potential “new” anti-inflammatory mediator in this process.

6. Summary of results

Paper I: Potential Anti-Inflammatory Role of Activin A in Acute Coronary Syndromes.

The aim of the study was to investigate whether the TGF-β superfamily member activin A could play a role in the immunopathogenesis of acute coronary disease.

- Patients with stable angina had raised activin A concentrations as assessed by protein levels in serum and mRNA levels in PBMC.
- In contrast to several reports on inflammatory cytokines, activin A levels were equal (serum) or even lower (PBMC) in unstable angina as compared to those with stable disease.
- In contrast to the enhanced expression of downstream activin A mediators (i.e., Smad3) in PBMC from stable angina patients as compared with healthy controls, no changes (i.e., Smad3) or even a down-regulation (Smad2) was seen in unstable disease. Likewise, the activin type II receptors, representing the primary ligand-binding protein, were down-regulated in unstable as compared to stable disease.
- Stable angina patients undergoing PCI showed a decrease in the activin A/follistatin ratio within 48 hours, suggesting down-regulatory effects on activin A activity during mechanically induced plaque rupture.
- While activin A dose-dependently suppressed the release of inflammatory cytokines from PBMC in stable and unstable angina, an opposite effect was found in healthy controls.

Paper II: Platelet-derived LIGHT induces inflammatory responses in endothelial cells and monocytes.

In paper II we sought to investigate whether the TNF superfamily member LIGHT was associated with platelets and if this cytokine could be involved in platelet-mediated inflammation.

- We found that platelet upon SFLLRN activation gradually released significantly amount of soluble LIGHT reaching a maximum after 120 minutes.

- The release of LIGHT involves GP IIb/IIIa-dependent mechanisms and action of metal-dependent proteases as well as intracellular processes such as actin polymerization.
- We also report that platelet-derived LIGHT is biologically active and can induce an inflammatory response in monocytes and particularly within endothelial cells measured as up-regulation of adhesion molecules E-selectin and VCAM-1 and release of the inflammatory chemokines IL-8 and MCP-1.
- We demonstrated that thrombus material, obtained at the site of plaque rupture in STEMI patients, contains platelet-associated LIGHT. Moreover, PCI, representing a mechanically induced plaque rupture, significantly increased plasma levels of LIGHT in stable angina patients, further suggesting that LIGHT-mediated inflammation also is operating *in vivo* within an inflamed and thrombotic vessel wall.

Paper III: Increased levels of Neutrophil-activating peptide-2 in acute coronary syndromes. Possible role of platelet-mediated vascular inflammation.

In this paper we investigated the role of the CXC chemokine neutrophil-activating peptide-2 (NAP-2) in CAD.

- Patients with stable and particularly those with unstable angina had markedly raised plasma levels of NAP-2 compared to controls, accompanied by increased expression of its receptor CXCR2 in monocytes.
- Even though NAP-2 is considered to be a predominantly platelet-derived chemokine, we found that PBMC released large amounts of NAP-2 upon stimulation with phytohemagglutinin (PHA), lipopolysaccharide (LPS), and the thrombin receptor agonist SFLLRN, with a particularly prominent response in unstable angina.
- By immunostaining, we showed that NAP-2 protein was localized in macrophages and vascular SMC of atherosclerotic carotid plaques and in monocytes and platelets of coronary thrombi from STEMI patients.
- *In vitro*, recombinant and platelet-derived NAP-2 increased the expression of adhesion molecules and chemokines in endothelial cells.
- While aspirin reduced NAP-2, statin therapy increased plasma levels of this chemokine with stimulating effects both on platelets and leukocytes.

Paper IV: Enhanced expression of the homeostatic chemokines CCL19 and CCL21 in clinical and experimental atherosclerosis: possible pathogenic role in plaque destabilization.

In this study we wanted to disclose any potential importance of the homeostatic chemokines CCL19 and CCL21 in CAD.

- We detected increased plasma levels of CCL19 and CCL21 in CAD patients compared to healthy controls, accompanied by decreased expression of their corresponding receptor CCR7 in PBMC, with the most marked changes in those with unstable disease.
- In contrast to the decreased CCR7 expression in circulating T cells from CAD patients, strong CCR7 immunostaining was seen in T cells within the atherosclerotic lesions of the $ApoE^{-/-}$ mice and in human atherosclerotic carotid plaques, accompanied by strong immunoreactivity of CCL19/CCL21 in macrophages and SMC within the atherosclerotic lesions.
- In macrophages, inflammatory stimuli and oxLDL down-regulated and cAMP up-regulated CCR7 expression.
- CCL19 and CCL21 were shown to induce an inflammatory phenotype in T cells and macrophages and increased MMP and tissue factor (TF) levels in the latter cell type.
- Aggressive statin therapy (i.e., atorvastatin 80 mg qd), but conventional statin therapy (i.e., simvastatin 20 mg qd), decreased plasma levels of CCL19/CCL21 levels and increased CCR7 expression in circulating $CD4^{+}CD3^{+}$ T cells in CAD patients.

Paper V: Increased levels and inflammatory effects of soluble CXCL16 in coronary artery disease - down-regulatory effects of statins.

The aim of this study was to elucidate the role of CXCL16 in CAD and study the ability of HMG-CoA reductase inhibitors (statins) to modulate CXCL 16 levels.

- Patients with stable and unstable angina had raised plasma levels of CXCL16 compared to controls, with no differences between these two groups of patients.
- Both conventional (simvastatin 20 mg qd) and aggressive (atorvastatin 80 mg qd) statin therapy significantly down-regulated plasma levels of CXCL16 during 6 months of

therapy.

- *In vitro*, atorvastatin significantly decreased the IL-1β-mediated release of CXCL16 from PBMC and endothelial cells.
- The attenuating effect of atorvastatin on the IL-1β-mediated release of CXCL16 in PBMC seems to involve inhibition of the protease ADAM10.
- Soluble CXCL16 enhanced the secretory potential in vascular SMC by increasing the release of IL-8, MCP-1 and MMPs.
- Soluble CXCL16 increased the release of IL-8 and MCP-1 in PBMC from healthy controls and particularly in cells from CAD patients.

7. Discussion

7.1 Methodological considerations

7.1.1 Individuals

Patients with verified CAD, defined either as stable effort angina NYHA class II or III and a positive exercise test, or as unstable angina defined as ischemic chest pain at rest within the preceding 48 hours (i.e., Braunwald´s class IIIBa) and transient ST-T segment depression and/or T-wave inversion, were studied in this thesis. The diagnosis of CAD was confirmed in all patients by coronary angiography showing at least 1 vessel disease (>50% narrowing of luminal diameter). According to standard procedures, the UAP patients had received heparin or low-molecular weight heparin before inclusion, but this medication had been discontinue >12 hours before blood sampling. Nevertheless, heparin therapy may influence the plasma/serum levels of cytokines and chemokines.[116]

To minimize confounding factors known to modulate inflammatory responses, only verified CAD patients without concomitant disease such as congestive heart failure, infections, lung diseases, cancers or autoimmune diseases were included in the studies.

7.1.2 Blood sampling and chemokine measuring in blood and cell culture supernatants

One of the main purposes of this thesis was to study cytokine levels in plasma/serum, mononuclear cells and platelets, obtained from CAD patients and healthy controls. Several factors related to blood collection and preparation of samples may influence the measured cytokine levels in plasma/serum and cells. First, contamination of collection tubes, buffers and media with endotoxins and microorganisms may activate the cells and induce inflammatory reactions.[117, 118] For *in vitro* studies, endotoxin levels were tested in all media, buffers and stimulant preparations used (all<10pg/mL). Second, time before storage, temperature during storage and freeze and thaw cycles may also influence cytokine levels.[119, 120] Therefore, in our experiments blood samples were immediately immersed on ice before processing, sera were kept frozen at -80°C, and thawed less than three times. Third, it has been reported that the recovery of cytokines from blood samples might be more optimal from plasma than from serum, and that EDTA should be preferred as an

anticoagulant as it seems to inhibit *ex vivo* cytokine production during plasma preparation.[121] Finally, in order to perform blood sampling in a way that ensured intact, non-activated platelets which at a later stage were able to respond to agonist stimulation, we used citrate as an anticoagulant which binds Ca^{2+} at a level that avoids plasma coagulation but allows platelet activation and aggregation due to agonist stimulation. It has been reported that citrated plasma with addition of the anti-platelet agent theophyllin, adenosine and dipyridamol (CTAD plasma) which may cause less degree of *ex vivo* platelet activation compared to citrated plasma alone, and would probably be a better alternative.[122] However, we did not experience problems with *ex vivo* platelet activation observed as minimal to non P-selectin expression and absent aggregation in samples with untreated patients.

7.1.3 Enzyme Linked Immunosorbent Assays (ELISAs)

Commercially available ELISAs were used for measuring the protein levels of cytokines in this study. The ELISA technique is based on the antibody sandwich principle, where a capture antibody specific to the protein of interest is bound to a microtiter plate to create a solid phase. After addition of the samples and standards, a second enzyme-conjugated detection antibody binds to a different epitope of the molecule being measured, completing the sandwich. The addition of a detection reagent (e.g., streptavidin-horseradish peroxidase) and a substrate solution (e.g., tatramethylbenzidine/hydrogen peroxide) leads to the development of color proportional to the amount of protein measured.

ELISAs are widely used as they are easy, time sparing and specific. However, some important limitations should be mentioned. First, the unique recognition profile of the antibodies will differ between kits from different manufacturers, making comparisons of results unreliable. Second, the presence of soluble cytokine receptors, cytokine antibodies and binding proteins in biological samples may affect the measured cytokine levels.[123] In the present study kits from the same manufacturer was used to be able to compare the results obtained. In most cases, compared data (e.g., patients versus controls) were run on the same microtiter plate to minimize run-to-run variability.

7.1.4 Analysis of gene expression

RNA was isolated using silica-gel based membranes (RNeasy columns), guanidium thiocynate-

phenol-cloroform as well as use of paramagnetic beads and the MagnaPure LC Robot. Notably, no comparisons were made between RNA extracted by different methods. All RNA samples were treated with DNase I to eliminate contamination of small amounts of DNA. RNA concentration and purity was evaluated by measuring the absorbency at 260/280 mm using a spectrophotometer. Only samples with high integrity and purity were used in our experiments (absorbance ratio >1.8).

To analyze the gene expression of specific transcripts we used real-time RT-PCR. Real-time RT-PCR is the most sensitive and time-saving method for detection of low-abundance mRNA. To correct for sample-to-sample variations, normalization of gene expression is performed against house-keeping (control) genes, the most commonly used being glyceraldehyde-3-phosphate-dehydrogenase (GAPDH), β-actin and 18S ribosomal RNA.[124] The control genes should ideally not be subject for any regulation in the tissue or cells investigated. In our experiments, house-keeping genes and the relative expression of these were stable between samples within the same experiment.

7.1.5 Cell isolation and culture

In this thesis several cellular experiments were performed, including studies in PBMC, HUVEC, vascular SMC, platelets and THP-1 macrophages. There is always a danger of unwanted *ex vivo* activation of cells during isolation procedures. Differences in blood sampling techniques or cell separation may be considered as important non-physiological *ex vivo* induction of cytokines. Accelerated blood flow during blood sampling and isolation of PBMC by density gradient centrifugation over Ficoll-Hypaque has been shown to induce up-regulation of the mRNA levels of several cytokines.[125] Accordingly, in this thesis a standardization of blood sampling and cell isolatiotion procedures was used in all experiments examining PBMC. After isolation the cells were immediately stored in liquid nitrogen (freshly isolated PBMC for gene analyses or used directly in cell cultures). Second, spontaneous activation of PBMC during culturing is well known, and monocytes appear to be the most sensible because of plastic adherence induced activation.[126] Third, platelets may easily become activated during isolation procedures.[127] In our experiments platelets were kept and cultured in autologous plasma (PRP), avoiding several centrifugation and resuspension steps compared with cultured in buffer. Finally, in all experiments involving cells, the results were compared when they were obtained during the same experiments, when the experiments had been run in parallel or when they involved paired observation of the same

patients, minimizing differences and misinterpretation of results due to *ex* vivo activation of cells. However, although *ex vivo* activation may contribute to enhanced chemokine mRNA and protein levels in several cell types, we believe that the differences between CAD patients and controls may be rather underestimated than overestimated in our experiments. It is also conceivable that inactive cells from healthy controls more easily become activated *ex vivo* than cells from patients already activated *in vivo*.[128]

7.2 Chemokines and atherogenesis

7.2.1 NAP-2: another CXCR2 ligand that is involved in atherogenesis and plaque destabilization

Chemokines are thought to be important actors in the inflammatory process characterizing atherogenesis and plaque destabilization. Thus, enhanced expression of various chemokines such as IL-8 and MCP-1 has been found within atherosclerotic plaques in humans, and targeted disruption of the genes for MCP-1, CCR2 (i.e., MCP-1 receptor), and CXCR2, which binds IL-8, significantly decrease atherosclerotic lesion formation in mice prone to develop atherosclerosis.[53-55] Moreover, some studies in mice models suggest that also other CXCR2 ligands (i.e., GRO-α) could be involved in atherogenesis at least partly by promoting monocyte arrest in inflamed endothelium.[129] However, while there are several reports on IL-8 in human CAD, few studies have examined the role of other CXCR2 ligands in human atherosclerotic disorders.

In paper III we report increased NAP-2 levels in angina patients with particularly high levels in those with unstable disease, accompanied by increased expression of its corresponding receptors (i.e., CXCR1 and CXCR2) on circulating monocytes. The relationship between raised NAP-2 levels and unstable disease was further supported by our immunohistochemical analyses showing strong NAP-2 immunostaining on platelets and monocytes/macrophages within thrombus material obtained at the site of plaque rupture in patients with STEMI undergoing PCI as well as in macrophages and SMC within symptomatic atherosclerotic carotid plaques. Our findings in paper III suggest that NAP-2 should be added to the list of CXCR2 ligands that could be involved in atherogenesis and plaque destabilization.

Chemokines can be produced by a variety of cells including leukocytes and endothelial cells, and recent studies suggest that also platelets contain a number of these chemotactic cytokines stored in their α-granules. Hence, upon activation, platelets release significant amounts of various

chemokines such as RANTES and PF4, promoting inflammation in adjacent leukocytes and endothelial cells.[76] Activated platelets also release large quantities of NAP-2 through proteolytic conversion from its precursors β-TG and connective tissue-activating proteinIII (CTAP III). Indeed, in paper III we show that platelets upon SFLLRN activation release large amount of NAP-2 in both angina patients and healthy controls. Among the chemokines stored and secreted by platelets, CXCL7 is the most abundant representative. In fact, by appearing in the serum at micromolar concentrations (1.6 to 4.8 μM), CXCL7 surpasses most other platelet-associated chemokines up to several orders of magnitude. CXCL7 consists of several molecular variants that differ in the length of their N-terminus such as b-TG, platelet basic protein (PBP), CTAPIII, and NAP-2, representing a highly inflammatory end product of CXCL7 proteolysis after platelet activation.[93] Although the actual ELISA used in paper III can not fully differ NAP-2 from other CXCL7 molecular variants, our finding strongly indicate enhanced levels of NAP-2 in CAD, representing an end-product of CXCL7 degradation and activation.

Though originally described as solely a platelet-derived chemokine, Waltz et al. detected NAP-2 in monocyte cultures after stimulation with LPS.[130] Recently, NAP-2 was found to be expressed in macrophages within advanced carotid atherosclerotic plaques.[131] In the present study, we extend these findings by showing that PBMC, THP-1 macrophages, and vascular SMC release NAP-2 upon various stimuli such as oxLDL and LPS, and notably, the release of NAP-2 from PBMC was particularly enhanced in cells from unstable angina patients further underscoring that NAP-2 is activated during unstable disease. Leukocyte-derived proteases have the capacity to cleave PBP and CTAP-III behind the single tyrosine residue present in CXCL7.[93] Although we cannot at present determine whether the elevated NAP-2 release in PBMC reflects increased synthesis or enhanced generation of NAP-2 from its precursors (i.e., enhanced protease activity) or both, our data clearly shows the ability of other cells than platelets to generate NAP-2. Moreover, an increase in NAP-2 was also seen at the mRNA level, suggesting that PBMC have the ability to produce NAP-2. Our finding of NAP-2 protein in macrophages and SMC within atherosclerotic carotid plaques and in monocytes of thrombus material obtained from the site of plaque rupture during MI, further support that these cells have the capacity to express NAP-2 and demonstrate that they do so in atherothrombosis.

NAP-2 virtually represents the only CXCL7 protein that may be addressed as a functionally typical chemokine, since it is the only variant capable of efficiently stimulating chemotactic

migration and thereby recruitment to inflammatory sites of its target cells. With regard to this, PBP and CTAPIII may be addressed as inactive precursors of NAP-2, although they exhibit a variety of other biological functions independently of proteolytical processing (e.g., effects on glucose metabolism and fibroblast mitogenic activity). In contrast, several studies support inflammatory effects of NAP-2 promoting recruitment and activation of granulocytes into inflamed tissue.[132] However, while most previous studies have focused on the effect of NAP-2 on granulocytes, we show in paper III that NAP-2 is a potent inducer of the adhesion molecules E-selectin and VCAM-1 as well as the chemokines MCP-1 and IL-8 in endothelial cells. This effect was induced not only by recombinant human (rh)NAP-2, but also by platelet-derived NAP-2. Furthermore, we found that monocytes from unstable angina patients showed enhanced chemotactic responses to NAP-2, suggesting that the increased CXCR2 expression in monocytes from these patients affects their functional responses. Previously, NAP-2 has been shown to promote monocyte arrest on inflamed endothelium under flow conditions, further supporting a role for NAP-2 in vascular inflammation.

NAP-2 binds to both CXCR1 (low-affinity) and CXCR2 (high-affinity). Although the ability of NAP-2 to function as an agonist for CXCR1, recent reports suggest that NAP-2 may address its two receptors in sequence and continue cell attraction through CXCR1 at high concentrations where CXCR2 has already undergone desensitization.[133] In paper III we found that neutralizing antibodies against CXCR1 and CXCR2 attenuated the NAP-2-induced increase in IL-8 and MCP-1 levels in HUVEC supernatants by 41% and 43%, respectively, indicating that this NAP-2-mediated effect involves ligation of both receptors. Nevertheless, although the exact mechanisms of action will have to be further investigated, our findings in paper III suggest that NAP-2 has the potential to induce an inflammatory response within the atherosclerotic plaque, and by its ability to promote leukocyte activation as well as expression of chemokines and adhesion molecules within the vessel wall, such a NAP-2-driven inflammation could ultimately lead to plaque rupture and acute coronary syndromes. Based on the high levels of NAP-2 in serum/plasma as well in the microenvironment within the atherosclerotic lesion, consisting of highly activated platelets and macrophages, both being relevant cellular sources of NAP-2, such NAP-2-mediated inflammation could clearly be of relevance also *in vivo* in CAD patients.

7.2.2 CXCL16: a transmembrane chemokine with pro-atherogenic properties

Chemokines expressed in inflammatory conditions were known to be secreted as soluble

molecules.[50] With the discovery of CX3CL1 and CXCL16, which are synthesized as transmembrane molecules and transported to the cell surface, this theory was abandoned.[134, 135] The scavenger receptor for phosphatidylserine and oxLDL (SR-PSOX) is a recently discovered macrophage receptor that mediates internalization of oxLDL and phosphatidylserine-coated particles such as apoptotic bodies.[136] Surprisingly, the sequence of SR-PSOX was found to be identical to the recently discovered chemokine, CXCL16. Along with these findings, its T cell expressed receptor, CXCR6 was discovered.[135] Moreover, CXCL16 was detected in macrophages *in vitro* and atherosclerotic lesions *in vivo*.[136-138] Hofnagel et al.[139] detected CXCL16 in cultured vascular SMC, cells that have later been shown also to express CXCR6. CXCL16 is the only known transmembrane CXC chemokine, existing in membrane-bound and soluble forms, and it fulfills functions of a T cell chemoattractant[140], a scavenger receptor for oxLDL as well as an adhesion molecule.[135, 136, 138, 141] Although this involvement in both inflammation and lipid metabolism could indicate a role in atherosclerosis, its role in atherosclerotic disorders is debated.

Increased levels of soluble CXCL16 have been reported in various inflammatory conditions such as rheumatoid arthritis and systemic lupus erythematosus.[142-144] It seems that cleavage of transmembrane chemokines is associated with the inflammatory cascade and, therefore, the soluble form of CXCL16 may serve as a stable marker of inflammation.[49] However, conflicting data exist on plasma levels of CXCL16 in CAD. While Sheikine found decreased plasma levels of CXCL16 in both stable and unstable angina[145], Lehrke reported increased plasma levels of this chemokine in a large population of CAD patients, particularly in those with unstable disease.[146] In paper V we report increased plasma levels of CXCL16 in CAD patients independent of co-morbidity such as diabetes and hypertension. The reasons for these discrepancies are at present not clear but could involve differences in blood sampling and storage protocols and differences in patient (e.g., different degree instability) and control characteristics. Nevertheless, Wuttge et al. have recently demonstrated increased expression of CXCL16 and CXCR6 in atherosclerotic plaques from humans and apolipoprotein-E-deficient mice.[138] Taken together, these data suggest increased expression of CXCL16 in human and murine atherosclerotic disease, both in circulation and within the atherosclerotic lesion.

The duality of CXCL16, existing in membrane bound and soluble forms, enables CXCL16 to both promote binding and adhesion of lymphocytes, oxLDL, and bacteria, as well as acting as a classical chemoattractant towards different leukocyte subsets, reflecting that the membrane-bound

and soluble form of CXCL16 possesses different biological functions.[140, 49] Much focus has been drawn against the function of the transmembrane form. However, while membrane-bound CXCL16, expressed on endothelial cells and macrophages within the vessel wall, has been linked to vascular inflammation by its ability to promote binding and adhesion of lymphocytes, soluble CXCL16 may also promote inflammatory responses, acting as a classical chemoattractant against various lymphocyte subsets.[49] Moreover, there are also some reports suggesting that soluble CXCL16 may have functions beyond that of leukocyte recruitment.[147, 148] Hence, CXCL16 has been found to enhance the activity of antigen-presenting cells involving toll-like receptor (TLR)9-related mechanisms.[149] Soluble CXCL16 has also been shown to promote SMC proliferation.[150] Herein we report that CXCL16-activated vascular SMC release inflammatory chemokines and MMPs, suggesting that CXCL16 may transform these cells from a contractile to a proliferative/secretory phenotype which is a hallmark of the vascular remodeling characterizing atherogenesis. Moreover, we found that soluble CXCL16 promoted IL-8 and MCP-1 release in PBMC, with a particularly marked response in CAD patients, further underscoring the relevance of CXCL16-mediated inflammation in atherogenesis. One might argue that the concentration of CXCL16 used in the *in vitro* experiments is not relevant to the *in vivo* situation. However, it is not inconceivable that similar concentrations may be found in the inflammatory microenvironment within an atherosclerotic plaque, consisting of several types of CXCL16 secreting cells (e.g., macrophages, SMC, and endothelial cells). The combination of CXCL16 with several other inflammatory cytokines operating within the atherosclerotic lesion may further enhance its inflammatory potential. Thus, based on our data in paper V, it is tempting to hypothesize that CXCL16 is not only a marker but also a mediator of inflammation in CAD patients

CXCL16 has been shown to be up-regulated in atherosclerotic plaques by the pro-atherogenic cytokine IFN-γ, potentially acting in a positive feedback loop to increase inflammation in the atherosclerotic lesion.[138] CXCL16 seems also to be mediator of the pro-atherogenic effects of IL-18. Since CXCL16 are induced by the Th1 master cytokine IFN-γ and preferentially act on Th1 cell subsets compared with Th2 cells, it has been suggested to further enhance Th1-associated inflammatory responses with potential pro-atherogenic consequences. In contrast to these findings, as well as our data in paper V, a recent murine study suggests that targeted disruption of CXCL16 accelerates atherosclerosis accompanied by increases macrophage recruitment to the aortic arch and elevated MCP-1 and TNF-α mRNA levels.[151] However, as the membrane-bound and soluble

form of CXCL16 seem to have different biological function, enhanced atherogenesis in CXCL16 knock-out mice may not necessarily argue against a pro-atherogenic effect of soluble CXCL16. *In vitro,* macrophages from CXCL16-deficient mice display reduced capacity to bind and internalize oxLDL. However, while this scavenger receptor function may account for the atheroprotective role of CXCL16, the inflammatory properties of CXCL16, and in particular of the soluble form lacking the ability to bind oxLDL, may counteract this beneficial effect. Also, substantial evidence indicates that CXCL16 may have constitutive functions such as promotion of cell survival and normal leukocyte recruitment. In the liver CXCL16/CXCR6 interaction confers to survival of patrolling NKT cells.[152] CXCL16 is also expressed by epithelial cells associated with gut lymphatics, where it may be involved in constitutive homing and positioning of T cells in the absence of inflammation.[153] Thus, while too much CXCL16 may be harmful, too little may not necessarily be beneficial. Interestingly, it has recently been shown that blocking CXCL16 actions by anti-sera limits the progression of glomerulonephritis[154], supporting a role for CXCL16 as a target for anti-inflammatory therapy. However, future studies will have to more precisely define the inflammatory and constitutive functions of CXCL16 as well as clarify the different effects of the membrane-bound as opposed to the soluble form of CXCL16 in inflammation.

Anti-TNF therapy[142] and the peroxisome proliferator-activated receptor-γ agonist pioglitazone[146] have previously been found to down-regulate serum levels of CXCL16 in patients with rheumatoid arthritis and metabolic syndrome, respectively. In paper V we show that statin therapy significantly reduces plasma levels of CXCL16 in CAD patients, independently of cholesterol lowering. Moreover, *in vitro* experiments showed the ability of atorvastatin to attenuate the release of CXCL16 in IL-1-activated endothelial cells and PBMC further supporting such a notion. CXCL16 is synthesized as a transmembrane molecule and transported to the cell surface. To date, no evidence that this form arise from differential splicing has been provided. Instead, the conversion of transmembrane into soluble CXCL16 has been attributed to a metalloproteolytic process. This cleavage can occur at the cell surface but it cannot be excluded that both chemokines are also cleaved in intracellular compartments. ADAM10 has been found to be a major regulator of CXCL16 shedding from its membrane-bound form.[146, 155] Our finding in paper V may suggest that atorvastatin attenuates the release of CXCL16 from PBMC by inhibiting ADAM10. Increased ADAM10 activity, resulting in increased release of soluble CXCL16 from the cell surface has been shown to be accompanied by down-regulation of the transmembrane CXCL16 form. However, in

paper V we found that the inhibition of CXCL16 release, most probably as a result of attenuated ADAM10 activity, was not associated with increased expression of CXCL16 on the cell surface. Although an atorvastatin-mediated inhibition of CXCL16 cleavage in intracellular compartments remain a potential explanation[49], these issues will have to be further investigated in forthcoming studies. Nevertheless, our findings further support the notion that statins possess anti-inflammatory properties partly independent of its lipid lowering effects. However, although ~80% of the angina patients used statins, they still had significantly raised plasma levels of CXCL16. Moreover, in paper III we showed that both aggressive (i.e., atorvastatin 80 mg qd) and conventional (simvastain 20 mg qd) significantly enhanced plasma levels of NAP-2 in CAD patients after 6 months of therapy, and such inflammatory effects of statins have also been reported by others.[156] Although we clearly do not argue against a beneficial role for statins in atherosclerotic disorders, these results suggest that other medication with another anti-inflammatory profile could also be of interest in these disorders.

7.2.3 CCL19 and CCL21: Inflammatory effects of homeostatic chemokines in CAD

In contrast to the above mentioned inflammatory chemokines, the homeostatic CCL19 and CCL21 are constitutively expressed within lymphoid tissues, and involved in maintaining homeostatic leukocyte trafficking and cell compartmentalization within these organs.[157] Secondary lymphoid organs orchestrate immune responses by optimizing encounters of lymphocytes with APC such as DC.[158, 159] The control of DC migration is pivotal for the initiation of cellular immune responses. DCs are professional APCs who reside in the periphery in an immature state. When activated with inflammatory stimuli, they undergo phenotypical and functional changes, including up-regulation of the chemokine receptor CCR7.[160] Activated DCs then home to the lymphoid organs, a migration governed with high precision by the chemokines CCL19 and CCL21, the two ligands for CCR7.[161, 162] Naive or memory T cells enter the lymph node using the same receptors. Once inside the lymph node, $CCR7^+$ DCs and T cells follow gradients of CCL19 and CCL21 in T cell zones to find one another. Thus, CCR7 and its ligands link innate and adaptive immunity through their effects on interactions between T cells and DCs.

In addition to their role in leukocyte and DC migration, recent studies have implied a potential role of CCL19/CCL21 in regulation of their immunological potential, possibly promoting inflammatory responses.[163, 164] CCL21 was also found to induce *de novo* formation of lymph node-

like structures with infiltration of lymphocytes in non-lymphoid tissue, further underscoring their inflammatory potential.[43, 44] Moreover, CCL19/CCL21/CCR7 have been implicated in the pathogenesis of various autoimmune and inflammatory disorders such as rheumatoid arthritis, diabetes mellitus, and inflammatory bowel disease.[44, 165, 166]

Based on their role in T cell homing into non-lymphoid tissue, which potentially could be an atherosclerotic plaque, along with their newly discovered involvement in inflammation, we in paper IV wanted to disclose a possible role of CCL19 and CCL21 as well as their common receptor CCR7 in CAD. By combining different approaches including clinical studies in CAD patients, *in vitro* studies, and *in vivo* experiments in $ApoE^{-/-}$ mice, we found an implication of these chemokines in CAD. We demonstrated increased CCL19/CCL21 expression in mouse and human atherosclerotic lesions as well as in plasma of CAD patients with particularly high levels in those with unstable disease. Interestingly, enhanced expression of the corresponding receptor CCR7 was detected in atherosclerotic plaque T cells, whereas circulating T cells from angina patients showed decreased CCR7 expression, primarily reflecting a down regulation in the percentage of $CCR7^{+}CD4^{+}$ T cells,. Although several non-mutually exclusive mechanisms may explain this phenomenon, we hypothesize that this pattern at least partly reflects a distribution of CCR7 positive T cells from peripheral blood to the inflamed atherosclerotic lesions during plaque progression. However, several issues will have to be further clarified in forthcoming studies. It has been suggested previously that the $CCR7^{-}$ T cell subset defines an antigen-experienced, tissue-homing (not lymph-node homing) memory T cell population with reduced proliferative capacity.[167] These T cells have been termed effector-memory T cells, as increased effector functions have also been described in $CCR7^{-}$ T cells, such as a strong expression of IFN-γ, perforin, and granzyme A.[168] Thus, it is possible that the decreased proportion of CCR7 negative T cells in peripheral blood in CAD may not necessarily be benificial, but rather represent a systemic inflammatory T cell phenotype. The recent observation that CCR7 is of importance for the function of regulatory T cells further support such a notion.

While we found no CCR7 expression in freshly isolated monocytes, THP-1 macrophages showed increased CCR7 expression. In paper IV we also showed that cAMP further enhances this up-regulation of CCR7 expression during macrophage differentiation. β-adrenergic receptor activation is an important stimulus for elevation of intracellular cAMP levels, and as CAD patients are known to have persistently increased catecholamine levels[169], this finding could be of

relevance also in relation to CAD. In fact, we found a CCR7-inducing effect in monocytes of the β-adrenergic receptor agonist isoproterenol, further supporting such a notion. Our findings could suggest an up till now unrecognized immunomodulatory potential of β-adrenergic receptor blockers, but if such mechanisms are operating *in vivo* in CAD patients with persistently activation of both the immune and the β-adrenergic systems and with desensitizing of the β-adrenergic receptors are far from clear. In contrast to the cAMP elevating agents, various inflammatory stimuli (i.e., TNFα as well as TLR2 and TLR4 agonists) down-regulated CCR7 expression in the early phase of macrophage differentiation although the effect of LPS was rather modest. These data illustrate the complex regulation of CCR7 expression in macrophages within an inflammatory lesion such as an atherosclerotic plaque, being exposed to both down-regulatory (i.e., some inflammatory stimuli as well as oxLDL) and enhancing (i.e., cAMP elevating agents such as prostaglandin E_2, which is abundant within atherosclerotic lesion, as well as an increased β-adrenergic tone). These counteracting effects on CCR7 expression in macrophages that may be operating within an inflammatory atheroclerotic lesions could perhaps explain the rather weak CCR7 immunostaining in macrophages within human atherosclerotic carotid plaques. In contrast to the effect on CCR7 expression in macrophages, cAMP elevating agents had no effects on CCR7 expression in T cells, further underscoring that the effect of the cAMP/protein kinase A type I system at least partly may differ between different cell types. Nevertheless, our data in paper IV illustrate the complex regulation of CCR7 expression during inflammation.

In addition to their ability to initiate and stimulate primary immune responses, CCL19 and CCl21 have recently been found to possess inflammatory properties as potent inducers of pro-inflammatory cytokines in DC supernatants with a secondary promotion of a Th1-like phenotype in adjacent T-cells.[164] Herein we show that CCL19 and particularly CCL21 induce inflammatory responses not only in T cells, but also in THP-1 macrophages by promoting the release of inflammatory cytokines. Moreover, and with relevance to plaque destabilization and thrombus formation, both CCL19 and CCL21 increased MMP activity and TF levels in THP-1 macrophages. If such mechanisms also are operating within the atherosclerotic lesion, they may in addition to promote an inflammatory Th1 phenotype, also induce a matrix degrading, inflammatory, and pro-thrombotic phenotype in CCR7 expressing macrophages. Based on the high levels of CCL19/CCL21 in angina patients, with particularly high levels in unstable disease, as well as the increased expression of these molecules in symptomatic human carotid plaques, these mediators

could potentially contribute to atherogenesis and plaque destabilization with subsequent thrombus formation and development of acute ischemic events. In fact, it is tempting to hypothesize that the combined feature of increased proportion of effector memory T cells in peripheral blood and increased interaction between CCL19/CCL21 and CCR7 expressing macrophages within the atherosclerotic lesion may result in both systemic and local inflammation in CAD. Two recent studies studies further support a role for CCL19/CCL21/CCR7 interaction in atherogenesis. First, Erbel et al. demonstrated that plaque tissues from patients with ischemic complications contained elevated levels of CCL19 and CCL21 primarily reflecting enhanced activation of dendritic cells within the atherosclerotic lesions.[170] Second, Trogan et al. showed that blocking experiments with neutralizing antibodies to CCL19 and CCL21 revealed that CCR7 was functionally required for plaque regression in apoE-deficient mice.[171]

Several clinical and experimental data indicate beneficial statin effects in addition to the lipid lowering activity particularly involving the inflammatory arm of atherogenesis.[172, 173] In line with the findings in paper V (i.e., CXL16), but in contrast to our finding in paper III showing up-regulation of NAP-2, such anti-inflammatory effects was also demonstrated in paper IV. However, whereas both aggressive (i.e., atorvastatin 80 mg qd) and conventional (simvastain 20 mg qd) significantly attenuated plasma levels of CXCL16 (paper V), only aggressive atorvastatin therapy significantly down-regulated plasma levels of CCL19/CCL21 and up-regulated CCR7 expression in PBMC, primarily reflecting an increase in $CCR7^+$ $CD4^+$ T cells. Whether this reflects a redistribution of T-cells from the atherosclerotic lesions to the circulation remains unclear. Whether this observation reflects redistribution from the inflamed atherosclerotic lesions to the circulation is at present not clear. However, two very recent papers have suggested that CCR7 may play an important role in emigration of T-cells from inflamed tissue with particularly pronounced effect on mobilization of the $CCR7^+CD4^+$ T-cells.[174, 175] Moreover, Yilmaz et al. have recently showed that statin pre-incubated dendritic cells exhibited an immature phenotype with a significantly lower expression of CCR7, suggesting that these medications also could modulate CCR7 expression in macrophages within the plaque.[176] Moreover, if the different effects of low dose simvastain and high dose atorvastain on CCL19/CCL21/CCR7 expression reflect drug- or dose-dependent differences or both should be further investigated. Nevertheless, some recent studies suggest that an intensive lipid-lowering statin regimen provides greater protection against cardiovascular events than does a standard regimen[177], and it is possible that the superiority of

high-dose atorvastain also involves more potent immunomodulatory effects of this medication. Future studies should also investigate if the anti-inflammatory potential differs between lipophilic and hydrophilic statins.

7.3 LIGHT: a "new" platelet-derived mediator of inflammation

Beyond the traditional role of platelets as mediators of haemostasis and thrombus formation, increasing evidence now suggests that activated platelets play a key role in inflammation. Hence, upon activation platelets release and express inflammatory mediators, induce an inflammatory response in adjacent leukocytes and endothelial cells, and respond with activation to several of the mediators produced by these cells. Such interactions between platelets and leukocytes/endothelial cells seem to play a pathogenic role in atherosclerosis as well as in other immune-mediated disorders. Platelets have upon activation been shown to secrete a number of inflammatory meditors such as PF-4, β-TG, RANTES, and GRO-α.[64, 72-74] Recently, much attention has been focused on the role of platelet-associated CD40L, a ligand in the TNF superfamily, in this inflammatory loop between platelets and other cells [64, 178], and these cells have also been shown to express TNF-related apoptosis-inducing ligand (TRAIL).[89] In paper II we identified **L**ymphotoxins, exhibits **I**nducible expression, and competes with herpes simplex virus (HSV) **G**lycoprotein for **H**erpes virus entry mediator (HVEM/TR2), a receptor expressed by **T** lymphytes (LIGHT), another member of the TNF superfamily, as a potential important mediator of platelet-mediated inflammation.

LIGHT signals through two different receptors; Herpes virus entry mediator (HVEM) and lymphotoxinβ receptor (LTβR). Studies in animal models suggest that LIGHT signaling pathways may be crucial for the development of various autoimmune disorders such as inflammatory bowel disease, nephritis, diabetes mellitus, and arthritis through effects on T cells and T cell homing.[179-181] While several studies have examined the pathogenic role of LIGHT in a variety of animal models[182, 183], there are few reports on the role of this cytokine in human disorders. However, enhanced LIGHT expression has recently been detected in human atherosclerotic plaques[184], and our group has shown elevated plasma levels of LIGHT in CAD patients with particularly high levels in those with unstable disease.[185] In paper II we found increased plasma levels of LIGHT in CAD patients undergoing PCI, a human *in vivo* model for mechanical induced plaque rupture, as

well as in thrombus material obtained at the site of plaque rupture in patients with acute MI. These finding suggest the involvement of LIGHT in atherogenesis and plaque destabilization in human.

LIGHT is expressed by activated T cells, monocytes, granulocytes, and immature dendritic cells, but until the present paper (paper II), it has not been identified in platelets. In paper II we demonstrated that LIGHT is secreted from platelets upon activation. In contrast to the rapid release of α-granule contents, activated platelets release LIGHT in a gradual and long-lasting manner, displaying a similar pattern as previously has been described for platelet-release of sCD40L.[89-91] As for the mechanism of LIGHT-release from platelets, our findings in paper II suggest that the GPIIb/IIIa complex is important for this process. Thus, Glanzman thromasthenia platelets and normal platelets treated with a fibrinogen-blocking GPIIb/IIIa antagonist, and thereby inhibiting platelet aggregation[90], showed markedly reduced activation-dependent LIGHT release. A similar pattern was also seen during GPIIb/IIIa inhibitory therapy *in vivo*, with a decrease in SFLLRN-mediated LIGHT-release *ex vivo* in PRP from unstabile angina patients receiving GPIIb/IIIa antagonist therapy as compared with those who did not (paper II). These findings may indicate that maximal release of LIGHT requires cell-to-cell contact and that this is prevented in Glanzman thromasthenia and in the presence of GPIIb/IIIa antagonists, or that an outside-in signaling due to binding of fibrinogen to the activated GPIIb/IIIa complex may be important in this respect. Cytochalasin D, which is known to inhibit actin polymerization and disrupt actin filaments, and therefore affect endocytotic and exocytotic transport processes, reduced the release of LIGHT from activated platelets. Further, the MMP inhibitor GM6001, which potentially can act both intracellularly and on the outer platelet membrane, and EDTA, which may neutralize metal-dependent enzymes on the outer membrane, both reduced the release of LIGHT from activated platelets (paper II). These results may implicate intracellular structures and reactions to be important for the release of LIGHT, and suggest that LIGHT, at least partly, is released from the platelets due to the actions of metal-dependent enzymes. Although the release of sCD40L and LIGHT during platelet activation show a similar long-lasting pattern as opposed to the rapid release (i.e., within 10 minutes after stimulation), differences between these TNF superfamily ligands may also exist. Thus, while platelet-bound CD40 seems to be involved in the regulation of sCD40L release, this seems not to be the case for the corresponding LIGHT receptors (i.e., HVEM and LTβR). Moreover, our group has recently showed that these ligands respond differently to prostacyclin therapy *in vivo* (Otterdal K, Aukrust P, Damås JK, unpublished data).

It has been shown that soluble LIGHT induces increased IL-8 secretion and growth arrest in melanoma cells[186], and Anand et al. showed that soluble LIGHT plays an important role in the pathogenesis of liver inflammation.[187] These findings indicate that soluble LIGHT is biologically active, and that recombinant soluble LIGHT can be used as an alternative to native LIGHT in *in vitro* experiments. In paper II we show that recombinant soluble LIGHT at 1-100 ng/mL is a potent inducer of the adhesion molecules E-selectin and VCAM-1 as well as the chemokines MCP-1 and IL-8 in endothelial cells. Importantly, these effects were not only seen in our experiments using recombinant LIGHT, but also with platelet-derived LIGHT. Thus, although the amount of platelet-derived LIGHT is relatively small as compared to, for example sCD40L (i.e., pg versus ng levels), neutralizing antibody against LIGHT significantly reduced the platelet-mediated enhancing effect on MCP-1 and IL-8 release in HUVEC, suggesting that platelet-derived soluble LIGHT is biologically active and possesses inflammatory properties. Heo et al. recently reported that LIGHT also had the ability to enhance monocytes activation[188], and our findings in paper II suggest that recombinant as well as platelet-derived LIGHT enhance the release of MCP-1 and IL-8 from monocytes, however, in a modest way as compared to HUVEC. While we previously have shown enhancing effects of recombinant CD40L on the release of IL-8 and MCP-1 only at concentrations above 1 μg/mL [189], similar effects of recombinant LIGHT was seen at a concentration of 1 ng/mL further underscoring that soluble LIGHT is a potent inducer of platelet-mediated inflammation.

Up-regulation of adhesion molecules and chemokines in endothelial cells is an important step in the recruitment and activation of leukocytes into inflamed tissue such as an atherosclerotic plaque. The LIGHT-mediated effects on endothelial cells as shown in paper II could therefore clearly be relevant to its potential role in atherogenesis. Moreover, Wei et al. demonstrated enhancing effects of LIGHT on macrophage migration as well as on SMC proliferation[190], and LIGHT has also been shown to increase the expression of TF and PAI-1 as well as to enhance thrombin formation and MMP activity in macrophages, transforming these cells into a pro-thrombotic and matrix degrading phenotype.[184, 185] Moreover, Scholz et al. have recently showed that LIGHT enhances lipid accumulation in oxLDL stimulated THP-1 macrophages possibly involving up-regulation of SR-A. The ability of LIGHT to modulate lipid metabolism was further supported in a recent study by Lo et al showing that dysregulation of LIGHT expression in T cells in mice resulted in hyperlipidemi at least partly by modulating the hepatic lipoprotein lipase activity.[191] Taken together, these observations indicate that LIGHT could serve as a molecular link

between lipid metabolism, inflammation, thrombus formation, and matrix degrading, features that all are present in atherosclerotic plaques. These findings also suggest that the raised LIGHT levels in CAD patients, seen both in plasma and within the atherosclerotic lesions, may represent important pathogenic processes in patients. Future studies should investigate if all these effects of LIGHT could be induced by relevant concentrations of platelet-derived LIGHT.

7.4 Activin A: a potential anti-imflammatory actor in CAD and plaque destabilization

As previously described, inflammation is a multifaceted process occurring at cellular, tissue, and systemic levels, mediated by the release of a range of cytokines. The balance between inflammatory (e.g., TNFα, IL-6,) and anti-inflammatory cytokines (e.g., IL-10) seems be of critical importance in the pathogenesis of plaque formation and destabilization, and we and others have suggested an inflammatory imbalance in unstable disease with "inadequately" raised IL-10 levels.[97] Our findings in paper I suggest that activin A could represent an additional anti-inflammatory actor in CAD.

Activin A is a member of the TGF-β superfamily and like TGF-β isoforms primarily signals via Smad2 and Smad3. Follistatin regulates activin A bioactivity by preventing activin from interacting with its signaling receptors, and this activin-binding protein is also a target of Smad2/3 signaling, representing a short-loop feedback system. We have recently shown that activin A may play a role in the development of heart failure.[192] Activin A has also been shown to be involved in inflammatory bowel disease[114], pulmonary fibrosis[193], asthma[194], inflammatory arthropathies[195], and has also reported to be rapidly released into circulation following endotoxin administration.[196] A potential role for activin A in atherosclerosis was first supported by its demonstration within atherosclerotic lesions.[197, 198] Later studies have emphasized a link between activin A and atherogenesis by showing that activin A could inhibit foam cell formation, induce a non-contractile SMC phenotype as well as prevent intimal vascular hyperplasia.[115, 199, 200] Based on these properties, it was conceivable to hypothesize a role for activin A in the pathogenesis of CAD and ACS. In paper I, we show that patients with CAD have elevated levels of activin A, but unlike the pattern for several inflammatory cytokines[81, 201, 202], only those with stable angina displayed enhanced gene expression of this cytokine in PBMC. Furthermore, in contrast to the enhanced expression of downstream activin A mediators (i.e., Smad3) in PBMC from stable angina patients

as compared with healthy controls, no changes (i.e., Smad3) or even a down-regulation (Smad2) was seen in unstable disease. Likewise, the activin type II receptors, representing the primary ligand-binding protein, were down-regulated in unstable as compared to stable disease. Thus, although activin A and related parameters seem to be up-regulated in CAD, they show a different pattern than several inflammatory cytokines, with no changes or even down-regulated levels in unstable disease. Our findings in paper I showing that a decrease in serum levels of activin A after a mechanically induced plaque rupture (i.e., PCI) was accompanied by increased levels of its natural inhibitor (i.e., follistatin), further support decreased activin A activity during plaque destabilization.

Activin A has previously been implicated as a stimulator in the production of inflammatory cytokines in macrophages[203] as well as an inducer of monocyte chemotaxis[204] and vascular SMC differentiation.[115, 199, 200] Activin A has also been reported to increase IL-6 and IL-8 expression during pregnancy.[205] However, activin A seems to have complex immunomodulatory properties and the dichotomy between pro-and anti-inflammatory actions appears to be a central aspect of activin A biology. Thus, although the inflammatory effects are well documented, anti-inflammatory actions have also been reported such as antagonizing effects of IL-6 in different cell types.[206] Activin A has also been shown to inhibit the IL-6-mediated secretion of acute phase proteins.[207] A major finding in paper I was that while activin A dose-dependently suppressed the release of inflammatory cytokines (i.e., IL-6, IL-8 and MIP-1α) from PBMC in stable and unstable angina, an opposite effect was found in healthy controls. The reason for these different responses in PBMC from angina patients and healthy controls is unclear at present, but may involve several factors such as different expression pattern of the activin A receptors and Smads. Feinberg et al. demonstrated that TGF-β inhibited chemokine expression through Smad-related pathways[208], and it is possible that the activin A-mediated inhibition of inflammatory cytokines may involve similar mechanisms. Moreover, it is well known the effect of various cytokines may largely depend on a different degree of cell pre-activation and the presence of co-activators and co-repressors, and such factors could clearly be involved in the apparently conflicting reports of immunomodulatory effects of activin A. Thus, while activin A could induce inflammatory responses PBMC from healthy controls, the stimulation of PBMC from angina, being pre-activated *in vivo* during their interactions with several inflammatory mediators, could result in down-regulation of inflammatory responses. We have recently shown that activin A induces anti-inflammatory and anti-oxidative

responses in endothelial cells that are pre-activated with IL-1β, but not in unstimulated cells (Smith C, Aukrust P, unpublished data), further supporting such a notion.

Activin A has been suggested to promote plaque stabilization by inhibiting foam cell formation through regulating scavenger receptor mRNA expression and by inducing a contractile, non-proliferative phenotype in cultured SMC.[115, 199, 200] In paper I we show potent anti-inflammatory effects of activin A in PBMC form angina patients, suggesting that an "inadequate rise" in activin A could contribute to an inappropriate inflammatory response in these patients. These findings may suggest an anti-inflammatory potential for this cytokine in CAD, and down-regulation of the activin system may be of importance in the progression from stable to unstable disease. Interestingly, the TGF-β type II receptor has been reported to be profoundly decreased in advanced atherosclerotic lesions, and disruption TGF-β signaling in T cells has been found to accelerate atherosclerosis. It is tempting to hypothesize that also dysregulated expression of activin A and its receptors, being a related member of the TGF-β superfamily, could contribute to atherogenesis and plaque destabilization.

8. Concluding remarks

- Stable angina patients were characterized by increased plasma levels and increased gene expression in PBMC of activin A. In contrast, the gene expression of activin A as well as the activin type II receptor was decreased in PBMC from unstable angina patients. A decreased ratio between activin A and its natural inhibitor follistatin was seen in patients undergoing PCI, further suggesting decreased activin A activity during plaque rupture. Activin A suppressed the release of inflammatory cytokines in PBMC from CAD patients, with an opposite response in cells from healthy controls. ***These findings suggest a potential anti-inflammatory and beneficial effect of activin A in atherogenesis and plaque destabilization.***
- Upon activation, platelets release soluble LIGHT, a member of the TNF superfamily, in a gradual manner, different from the rapid release from α-granules. The release involves GP IIb/IIIa dependent mechanisms and action of metal-dependent proteases as well as intracellular processes such as actin polymerization. Platelet-derived LIGHT is biologically active and induces inflammatory responses in monocytes and in particular in endothelial cells. Thrombus materials, obtained at the site of plaque rupture in STEMI patients, contains platelet-associated LIGHT suggesting that LIGHT-mediated inflammation also is operating *in vivo*. ***Our findings suggest that LIGHT should be added to the list of mediators that are involved in platelet-mediated inflammation, potentially contributing to vascular inflammation in atherosclerotic disorders.***
- CAD patients had raised plasma levels of NAP-2 with particularly high concentrations in those with unstable disease, accompanied by increased expression of its receptor CXCR2 in monocytes. Even though NAP-2 is considered to be a predominantly platelet-derived chemokine, PBMC were found to release large amounts of NAP-2 upon stimulation, in particular in CAD patients. NAP-2 protein was localized to macrophages and vascular SMC of atherosclerotic carotid plaques and in monocytes and platelets of coronary thrombi of STEMI patients. *In vitro*, recombinant and platelet-derived NAP-2 increased the expression of adhesion molecules and chemokines in endothelial cells. ***While there are several reports on IL-8 in human CAD, our data suggest that another CCR2 ligand (i.e., NAP-2) has the potential to induce inflammatory responses within the atherosclerotic plaque, and such a NAP-2-driven inflammation could ultimately lead to plaque rupture and ACS.***
- CAD patients had increased plasma levels of CCL19 and CCL21, accompanied by decreased expression of their common receptor CCR7 in PBMC, with the most marked changes in those with unstable disease. In contrast to the decreased CCR7 expression in circulating T cells from CAD patients, strong CCR7 immunostaining was seen in T cells within the atherosclerotic

lesions of both murine and human atherosclerosis, accompanied by strong immunoreactivity of CCL19 and CCL21 in macrophages and vascular SMC within the atherosclerotic plaques. *In vitro*, CCL19 and CCL21 were shown to induce an inflammatory, matrix degrading, and pro-thrombotic phenotype in macrophages. ***The abnormal regulation of CCL19 and CCL21 and their common receptor CCR7 in atherosclerosis could contribute to disease progression by recruiting T cells to the atherosclerotic lesions and by promoting inflammatory responses in T cells and macrophages within the plaque.***

- CAD patients had raised plasma concentrations of the transmembrane chemokine CXCL16, and these levels were down-regulated by statins both *in vivo* and *in vitro*, at least partly involving inhibition of the protease ADAM10. CXCL16 induced the production of inflammatory chemokines in PBMC, most prominently in CAD patients, and enhanced the secretory potential of inflammatory mediators in vascular SMC. ***Our findings suggest that CXCL16 could be linked to atherogenesis not only as a marker of inflammation, but also as a potential inflammatory mediator.***

Our findings further underscore the role of inflammation in the pathogenesis of atherosclerosis and plaque destabilization, involving interactions between platelets, endothelial cells, macrophages, T cells, and vascular SMC. Our findings also suggest that while NAP-2, CXCL16, CCL19, CCL21, and LIGHT could enhance these inflammatory interactions, activin A could attenuate these processes. Future studies should further characterize the pathogenic role and the relative importance of these mediators, with the ambitions to delineate novel therapeutic targets possibly leading to new treatment modalities in atherosclerotic disorders.

9. References

1. Davies MJ. The composition of coronary-artery plaques. N Engl J Med. 1997;336:1312-1314.

2. Lusis AJ. Atherosclerosis. Nature. 2000;407:233-241.

3. Libby P. Current concepts of the pathogenesis of the acute coronary syndromes. Circulation. 2001;104:365-372.

4. Falk E, Shah PK, Fuster V. Coronary plaque disruption. Circulation. 1995;92:657-671.

5. Farb A, Burke AP, Tang AL, Liang Y, Mannan P, Smialek J, Virmani R. Coronary plaque erosion without rupture into a lipid core: A frequent cause of coronary thrombosis in sudden coronary death. Circulation. 1996;93:1354-1363.

6. Hansson GK. Immune mechanisms in atherosclerosis. Arterioscler Thromb Vasc Biol. 2001;21:1876-1890.

7. Hansson GK, Libby P. The immune response in atherosclerosis: A double-edged sword. Nat Rev Immunol. 2006;6:508-519.

8. Zernecke A, Weber C. Inflammatory mediators in atherosclerotic vascular disease. Basic Res Cardiol. 2005;100:93-101.

9. Glass CK, Witztum JL. Atherosclerosis: The road ahead. Cell. 2001;104:503-516.

10. Libby P. Coronary artery injury and the biology of atherosclerosis: Inflammation, thrombosis, and stabilization. Am J Cardiol. 2000;86:3J-8J.

11. Libby P. Inflammation in atherosclerosis. Nature. 2002;420:868-874.

12. Libby P, Ridker PM, Maseri A. Inflammation and atherosclerosis. Circulation. 2002;105:1135-1143.

13. Rabbani R, Topol EJ. Strategies to achieve coronary arterial plaque stabilization. Cardiovasc Res. 1999;41:402-417.

14. Ross R. Atherosclerosis -- An inflammatory disease. N Engl J Med. 1999;340:115-126.

15. Tedgui A, Mallat Z. Cytokines in atherosclerosis: Pathogenic and regulatory pathways. Physiol Rev. 2006;86:515-581.

16. Hackett D, Davies G, Maseri A. Pre-existing coronary stenoses in patients with first myocardial infarction are not necessarily severe. Eur Heart J. 1988;9:1317-1323.

17. Blankenhorn DH, Hodis HN. George Lyman Duff Memorial Lecture. Arterial imaging and atherosclerosis reversal. Arterioscler Thromb. 1994;14:177-192.

18. Brown BG, Zhao XQ, Sacco DE, Albers JJ. Lipid lowering and plaque regression. New insights into prevention of plaque disruption and clinical events in coronary disease. Circulation. 1993;87:1781-1791.

19. Chen L, Chester MR, Crook R, Kaski JC. Differential progression of complex culprit stenoses in patients with stable and unstable angina pectoris. J Am Coll Cardiol. 1996;28:597-603.

20. Goldstein JA, Demetriou D, Grines CL, Pica M, Shoukfeh M, O'Neill WW. Multiple complex coronary plaques in patients with acute myocardial infarction. N Engl J Med. 2000;343:915-922.

21. Guazzi MD, Bussotti M, Grancini L, De Cesare N, Guazzi M, Pera IL, Loaldi A. Evidence of multifocal activity of coronary disease in patients with acute myocardial infarction.

Circulation. 1997;96:1145-1151.
22. Hansson GK. Inflammation, atherosclerosis, and coronary artery disease. N Engl J Med. 2005;352:1685-1695.
23. Boisvert WA. Modulation of atherogenesis by chemokines. Trends Cardiovasc Med. 2004;14:161-165.
24. Zhou X, Paulsson G, Stemme S, Hansson GK. Hypercholesterolemia is associated with a T helper (Th) 1/Th2 switch of the autoimmune response in atherosclerotic apo E-knockout mice. J Clin Invest. 1998;101:1717-1725.
25. Stemme S, Holm J, Hansson GK. T lymphocytes in human atherosclerotic plaques are memory cells expressing CD45RO and the integrin VLA-1. Arterioscler Thromb. 1992;12:206-211.
26. Mazzone A, De Servi S, Ricevuti G, Mazzucchelli I, Fossati G, Pasotti D, Bramucci E, Angoli L, Marsico F, Specchia G, et al. Increased expression of neutrophil and monocyte adhesion molecules in unstable coronary artery disease. Circulation. 1993;88:358-363.
27. Serneri GG AR, Gori AM, Attanasio M, Martini F, Giusti B, Dabizzi P, Poggesi L, Modesti PA, Trotta F, et al. Transient intermittent lymphocyte activation is responsible for the instability of angina. Circulation. 1992;86:790-797.
28. Hansson GK HJ, Jonasson L. Detection of activated T lymphocytes in the human atherosclerotic plaque. Am J Pathol. 1989;135:169-175.
29. Libby P, Geng YJ, Aikawa M, Schoenbeck U, Mach F, Clinton SK, Sukhova GK, Lee RT. Macrophages and atherosclerotic plaque stability. Curr Opin Lipidol. 1996;7:330-335.
30. van der Wal AC, Becker AE, van der Loos CM, Das PK. Site of intimal rupture or erosion of thrombosed coronary atherosclerotic plaques is characterized by an inflammatory process irrespective of the dominant plaque morphology. Circulation. 1994;89:36-44.
31. Buffon A, Biasucci LM, Liuzzo G, D'Onofrio G, Crea F, Maseri A. Widespread coronary inflammation in unstable angina. N Engl J Med. 2002;347:5-12.
32. Aukrust P, Yndestad A, Smith C, Sandberg WJ, Ueland T, Waehre T, Halvorsen B, Gullestad L, Damås JK. Potential role for immunomodulatory therapy in atherosclerotic plaque stabilisation. Expert Opin Pharmacother. 2005;6:2169-2180.
33. Ridker PM, Cushman M, Stampfer MJ, Tracy RP, Hennekens CH. Inflammation, aspirin, and the risk of cardiovascular disease in apparently healthy men. N Engl J Med. 1997;336:973-979.
34. Ridker PM, Hennekens CH, Buring JE, Rifai N. C-reactive protein and other markers of inflammation in the prediction of cardiovascular disease in women. N Engl J Med. 2000;342:836-843.
35. Adams DH, Rlloyd A. Chemokines: Leucocyte recruitment and activation cytokines. The Lancet. 1997;349:490-495.
36. Baggiolini M, Dewald B, Moser B. Human chemokines: An update. Annu Rev Immunol. 1997;15:675-705.
37. Slettenaar VIF, Wilson JL. The chemokine network: A target in cancer biology? Adv Drug Deliv Rev. 2006;58:962-974.
38. Luster AD. Chemokines -- chemotactic cytokines that mediate inflammation. N Engl J Med. 1998;338:436-445.
39. Ward SG, Bacon K, Westwick J. Chemokines and T lymphocytes: More than an attraction. Immunity. 1998;9:1-11.
40. O'Garra A, McEvoy LM, Zlotnik A. T-cell subsets: Chemokine receptors guide the way.

Curr Biol. 1998;8:R646-R649.
41. Horuk R. Chemokine receptors. Cytokine Growth Factor Rev. 2001;12:313-335.
42. Baggiolini M. Chemokines and leukocyte traffic. Nature. 1998;392:565-568.
43. Ashour AE, Turnquist HR, Singh RK, Talmadge JE, Solheim JC. CCL21-induced immune cell infiltration. Int Immunopharmacol. 2007;7:272-276.
44. Fan L, Reilly CR, Luo Y, Dorf ME, Lo D. Cutting edge: Ectopic expression of the chemokine TCA4/SLC is sufficient to trigger lymphoid neogenesis. J Immunol. 2000;164:3955-3959.
45. Hansson GK. Inflammation, atherosclerosis, and coronary artery disease. N Engl J Med. 2005;352:1685-1695.
46. Sallusto F, Mackay CR, Lanzavecchia A. The role of chemokine receptors in primary, effector, and memory immune responses. Annu Rev Immunol. 2000;18:593-620.
47. Remick DG. Interleukin-8. Crit Care Med. 2005;33:S466-467.
48. Weber C, Schober A, Zernecke A. Chemokines: Key regulators of mononuclear cell recruitment in atherosclerotic vascular disease. Arterioscler Thromb Vasc Biol. 2004;24:1997-2008.
49. Ludwig A, Weber C. Transmembrane chemokines: Versatile 'special agents' in vascular inflammation. Thromb Haemost. 2007;97:694-703.
50. Charo IF, Ransohoff RM. The many roles of chemokines and chemokine receptors in inflammation. N Engl J Med. 2006;354:610-621.
51. Schecter AD, Berman AB, Taubman MB. Chemokine receptors in vascular smooth muscle. Microcirculation. 2003;10:265 - 272.
52. Weber C. Platelets and chemokines in atherosclerosis: Partners in crime. Circ Res. 2005;96:612-616.
53. Boisvert WA, Santiago R, Curtiss LK, Terkeltaub RA. A leukocyte homologue of the IL-8 receptor CXCR-2 mediates the accumulation of macrophages in atherosclerotic lesions of LDL receptor-deficient mice. J Clin Invest. 1998;101:353-363.
54. Boring L, Gosling J, Cleary M, Charo IF. Decreased lesion formation in CCR2-/- mice reveals a role for chemokines in the initiation of atherosclerosis. Nature. 1998;394:894-897.
55. Gu L, Okada Y, Clinton SK, Gerard C, Sukhova GK, Libby P, Rollins BJ. Absence of monocyte chemoattractant protein-1 reduces atherosclerosis in low density lipoprotein receptor-deficient mice. Mol Cell. 1998;2:275-281.
56. Charo IF, Taubman MB. Chemokines in the pathogenesis of vascular disease. Circ Res. 2004;95:858-866.
57. Haque NS, Fallon JT, Pan JJ, Taubman MB, Harpel PC. Chemokine receptor-8 (CCR8) mediates human vascular smooth muscle cell chemotaxis and metalloproteinase-2 secretion. Blood. 2004;103:1296-1304.
58. Kodali R, Hajjou M, Berman AB, Bansal MB, Zhang S, Pan JJ, Schecter AD. Chemokines induce matrix metalloproteinase-2 through activation of epidermal growth factor receptor in arterial smooth muscle cells. Cardiovasc Res. 2006;69:706-715.
59. Moreau M, Brocheriou I, Petit L, Ninio E, Chapman MJ, Rouis M. Interleukin-8 mediates downregulation of tissue inhibitor of metalloproteinase-1 expression in cholesterol-loaded human macrophages: Relevance to stability of atherosclerotic plaque. Circulation.1999;99:420-426.
60. Moulton KS, Vakili K, Zurakowski D, Soliman M, Butterfield C, Sylvin E, Lo KM, Gillies

S, Javaherian K, Folkman J. Inhibition of plaque neovascularization reduces macrophage accumulation and progression of advanced atherosclerosis. Proc Natl Acad Sci U S A. 2003;100:4736-4741.

61. Schecter AD, Rollins BJ, Zhang YJ, Charo IF, Fallon JT, Rossikhina M, Giesen PLA, Nemerson Y, Taubman MB. Tissue factor is induced by monocyte chemoattractant protein-1 in human aortic smooth muscle and THP-1 cells. J Biol Chem. 1997;272:28568-28573.

62. Aukrust P, Berge RK, Ueland T, Aaser E, Damås JK, Wikeby L, Brunsvig A, Muller F, Forfang K, Frøland SS, Gullestad L. Interaction between chemokines and oxidative stress: Possible pathogenic role in acute coronary syndromes. J Am Coll Cardiol. 2001;37:485-491.

63. Furie B, Furie BC, Flaumenhaft R. A journey with platelet P-selectin: The molecular basis of granule secretion, signalling and cell adhesion. Thromb Haemost. 2001;86:214-221.

64. Henn V, Slupsky JR, Grafe M, Anagnostopoulos I, Forster R, Muller-Berghaus G, Kroczek RA. CD40 ligand on activated platelets triggers an inflammatory reaction of endothelial cells. Nature. 1998;391:591-594.

65. Kaneider NC, Kaser A, Tilg H, Ricevuti G, Wiedermann CJ. CD40 ligand-dependent maturation of human monocyte-derived dendritic cells by activated platelets. Int J Immunopathol Pharmacol. 2003;16:225-231.

66. Shattil SJ. Signaling through platelet integrin alpha IIb beta 3: Inside-out, outside-in, and sideways. Thromb Haemost. 1999;82:318-325.

67. Solum NO. Procoagulant expression in platelets and defects leading to clinical disorders. Arterioscler Thromb Vasc Biol. 1999;19:2841-2846.

68. Mann KG, Nesheim ME, Church WR, Haley P, Krishnaswamy S. Surface-dependent reactions of the vitamin K-dependent enzyme complexes. Blood. 1990;76:1-16.

69. Mosesson MW. Fibrinogen and fibrin structure and functions. J Thromb Haemost 2005;3:1894-1904.

70. Nieuwland R, Berckmans RJ, Rotteveel-Eijkman RC, Maquelin KN, Roozendaal KJ, Jansen PGM, Have Kt, Eijsman L, Hack CE, Sturk A. Cell-derived microparticles generated in patients during cardiopulmonary bypass are highly procoagulant. Circulation. 1997;96:3534-3541.

71. Ogura H, Kawasaki T, Tanaka H, Koh T, Tanaka R, Ozeki Y, Hosotsubo H, Kuwagata Y, Shimazu T, Sugimoto H. Activated platelets enhance microparticle formation and platelet-leukocyte interaction in severe trauma and sepsis. J Trauma. 2001;50:801-809.

72. Boehlen F, Clemetson KJ. Platelet chemokines and their receptors: What is their relevance to platelet storage and transfusion practice? Transfus Med. 2001;11:403-417.

73. Brandt E, Ludwig A, Petersen F, Flad H-D. Platelet-derived CXC chemokines: Old players in new games. Immunological Reviews. 2000;177:204-216.

74. Power CA, Clemetson JM, Clemetson KJ, Wells TNC. Chemokine and chemokine receptor mRNA expression in human platelets. Cytokine. 1995;7:479-482.

75. Deuel TF, Senior RM, Chang D, Griffin GL, Heinrikson RL, Kaiser ET. Platelet factor 4 is chemotactic for neutrophils and monocytes. Proc Natl Acad Sci U S A. 1981;78:4584-4587.

76. Weyrich AS, Zimmerman GA. Platelets: Signaling cells in the immune continuum. Trends Immunol. 2004;25:489-495.

77. Gawaz M, Brand K, Dickfeld T, Pogatsa-Murray G, Page S, Bogner C, Koch W, Schomig A, Neumann FJ. Platelets induce alterations of chemotactic and adhesive properties of

endothelial cells mediated through an interleukin-1-dependent mechanism. Implications for atherogenesis. Atherosclerosis. 2000;148:75-85.
78. Loppnow H, Bil R, Hirt S, Schonbeck U, Herzberg M, Werdan K, Theodor Rietschel E, Brandt E, Flad HD. Platelet-derived interleukin-1 induces cytokine production, but not proliferation of human vascular smooth muscle cells. Blood. 1998;91:134-141.
79. Clemetson KJ, Clemetson JM, Proudfoot AEI, Power CA, Baggiolini M, Wells TNC. Functional expression of CCR1, CCR3, CCR4, and CXCR4 chemokine receptors on human platelets. Blood. 2000;96:4046-4054.
80. Schönbeck U, Libby P. The CD40/CD154 receptor/ligand dyad. Cell Mol Life Sci. 2001;58:4-43.
81. Aukrust P, Muller F, Ueland T, Berget T, Aaser E, Brunsvig A, Solum NO, Forfang K, Frøland SS, Gullestad L. Enhanced levels of soluble and membrane-bound CD40 ligand in patients with unstable angina. Possible reflection of T lymphocyte and platelet involvement in the pathogenesis of acute coronary syndromes. Circulation. 1999;100:614-620.
82. Schonbeck U, Varo N, Libby P, Buring J, Ridker PM. Soluble CD40L and cardiovascular risk in women. Circulation. 2001;104:2266-2268.
83. Mach F, Schonbeck U, Sukhova GK, Atkinson E, Libby P. Reduction of atherosclerosis in mice by inhibition of CD40 signalling. Nature. 1998;394:200-203.
84. Mach F, Schonbeck U, Sukhova GK, Bourcier T, Bonnefoy JY, Pober JS, Libby P. Functional CD40 ligand is expressed on human vascular endothelial cells, smooth muscle cells, and macrophages: Implications for CD40-CD40 ligand signaling in atherosclerosis. Proc Natl Acad Sci U S A. 1997;94:1931-1936.
85. Schonbeck U, Mach F, Sukhova GK, Atkinson E, Levesque E, Herman M, Graber P, Basset P, Libby P. Expression of stromelysin-3 in atherosclerotic lesions: Regulation via CD40-CD40 ligand signaling in vitro and in vivo. J Exp Med. 1999;189:843-853.
86. Lutgens E, Gorelik L, Daemen MJ, de Muinck ED, Grewal IS, Koteliansky VE, Flavell RA. Requirement for CD154 in the progression of atherosclerosis. Nat Med. 1999;5:1313-1316.
87. Lutgens E, Cleutjens KBJM, Heeneman S, Koteliansky VE, Burkly LC, Daemen MJAP. Both early and delayed anti-CD40L antibody treatment induces a stable plaque phenotype. Proc Natl Acad Sci U S A. 2000;97:7464-7469.
88. Jin Y, Nonoyama S, Morio T, Imai K, Ochs HD, Mizutani S. Characterization of soluble CD40 ligand released from human activated platelets. J Med Dent Sci. 2001;48:23-27.
89. Crist SA, Elzey BD, Ludwig AT, Griffith TS, Staack JB, Lentz SR, Ratliff TL. Expression of TNF-related apoptosis-inducing ligand (TRAIL) in megakaryocytes and platelets. Experimental Hematology. 2004;32:1073-1081.
90. Nannizzi-Alaimo L, Alves VL, Phillips DR. Inhibitory effects of glycoprotein IIb/IIIa antagonists and aspirin on the release of soluble CD40 ligand during platelet stimulation. Circulation. 2003;107:1123-1128.
91. Furman MI, Krueger LA, Linden MD, Barnard MR, Frelinger AL, Michelson AD. Release of soluble CD40L from platelets is regulated by glycoprotein IIb/IIIa and actin polymerization. J Am Coll Cardiol. 2004;43:2319-2325.
92. Aukrust P, Waehre T, Damås JK, Gullestad L, Solum NO. Inflammatory role of platelets in acute coronary syndromes. Heart. 2001;86:605-606.
93. von Hundelshausen P, Weber C. Platelets as immune cells: Bridging inflammation and cardiovascular disease. Circ Res. 2007;100:27-40.

94. Gawaz M, Langer H, May AE. Platelets in inflammation and atherogenesis. J Clin Invest. 2005;115:3378-3384.
95. Waehre T, Damås JK, Yndestad A, Tasken K, Pedersen TM, Smith C, Halvorsen B, Frøland SS, Solum NO, Aukrust P. Effect of activated platelets on expression of cytokines in peripheral blood mononuclear cells - potential role of prostaglandin E2. Thromb Haemost. 2004;92:1358-1367.
96. Smith DA, Irving SD, Sheldon J, Cole D, Kaski JC. Serum levels of the antiinflammatory cytokine interleukin-10 are decreased in patients with unstable angina. Circulation. 2001;104:746-749.
97. Waehre T, Halvorsen B, Damås JK, Yndestad A, Brosstad F, Gullestad L, Kjekshus J, Frøland SS, Aukrust P. Inflammatory imbalance between IL-10 and TNFalpha in unstable angina potential plaque stabilizing effects of IL-10. Eur J Clin Invest. 2002;32:803-810.
98. Heeschen C, Dimmeler S, Hamm CW, Fichtlscherer S, Boersma E, Simoons ML, Zeiher AM, for the CSI. Serum level of the antiinflammatory cytokine interleukin-10 is an important prognostic determinant in patients with acute coronary syndromes. Circulation. 2003;107:2109-2114.
99. Pinderski Oslund LJ, Hedrick CC, Olvera T, Hagenbaugh A, Territo M, Berliner JA, Fyfe AI. Interleukin-10 blocks atherosclerotic events in vitro and in vivo. Arterioscler Thromb Vasc Biol. 1999;19:2847-2853.
100. Moore KW, de Waal Malefyt R, Coffman RL, O'Garra A. Interleukin-10 and the interleukin-10 receptor. Annu Rev Immunol. 2001;19:683-765.
101. Mallat Z, Gojova A, Marchiol-Fournigault C, Esposito B, Kamate C, Merval R, Fradelizi D, Tedgui A. Inhibition of transforming growth factor-{beta} signaling accelerates atherosclerosis and induces an unstable plaque phenotype in mice. Circ Res. 2001;89:930-934.
102. Mallat Z, Besnard S, Duriez M, Deleuze V, Emmanuel F, Bureau MF, Soubrier F, Esposito B, Duez H, Fievet C, Staels B, Duverger N, Scherman D, Tedgui A. Protective role of interleukin-10 in atherosclerosis. Circ Res. 1999;85:17e-24.
103. Terkeltaub RA. IL-10: An "immunologic scalpel" for atherosclerosis? Arterioscler Thromb Vasc Biol. 1999;19:2823-2825.
104. Robertson AKL, Rudling M, Zhou X, Gorelik L, Flavell RA, Hansson GK. Disruption of TGF-{beta} signaling in T cells accelerates atherosclerosis. J Clin Invest. 2003;112:1342-1350.
105. Derynck R, Zhang YE. Smad-dependent and Smad-independent pathways in TGF-[beta] family signalling. Nature. 2003;425:577-584.
106. Piek E, Heldin CH, Ten Dijke P. Specificity, diversity, and regulation in TGF-beta superfamily signaling. Faseb J. 1999;13:2105-2124.
107. Letterio JJ, Roberts AB. Transforming growth factor-beta1-deficient mice: Identification of isoform-specific activities in vivo. J Leukoc Biol. 1996;59:769-774.
108. Letterio JJ, Roberts AB. Regulation of immune responses by TGF-{beta}. Annu Rev Immunol. 1998;16:137-161.
109. Ruscetti FW, Palladino MA. Transforming growth factor-beta and the immune system. Prog Growth Factor Res. 1991;3:159-175.
110. Wahl SM. Transforming growth factor beta: The good, the bad, and the ugly. J Exp Med. 1994;180:1587-1590.
111. Toms C, Powrie F. Control of intestinal inflammation by regulatory T cells. Microbes

Infect. 2001;3:929-935.
112. Border WA, Noble NA. Transforming growth factor {beta} in tissue fibrosis. N Engl J Med. 1994;331:1286-1292.
113. Singh NN, Ramji DP. The role of transforming growth factor-[beta] in atherosclerosis. Cytokine Growth Factor Rev. 2006;17:487-499.
114. Hübner G, Brauchle M, Gregor M, Werner S. Activin A: A novel player and inflammatory marker in inflammatory bowel disease? Lab Invest. 1997;77:311-318.
115. Kozaki K, Akishita M, Eto M, Yoshizumi M, Toba K, Inoue S, Ishikawa M, Hashimoto M, Kodama T, Yamada N, Orimo H, Ouchi Y. Role of activin-A and follistatin in foam cell formation of THP-1 macrophages. Arterioscler Thromb Vasc Biol. 1997;17:2389-2394.
116. Seidel C, Hjorth-Hansen H, Bendz B, Borset M, Sandset PM, Hansen J-B, Sundan A, Waage A. Hepatocyte growth factor in serum after injection of unfractionated and low molecular weight heparin in healthy individuals. Br J Haematol. 1999;105:641-647.
117. Leroux-Roels G, Offner F, Philippe J, Vermeulen A. Influence of blood-collecting systems on concentrations of tumor necrosis factor in serum and plasma. Clin Chem. 1988;34:2373-2374.
118. Riches P, Gooding R, Millar BC, Rowbottom AW. Influence of collection and separation of blood samples on plasma IL-1, IL-6 and TNF-[alpha] concentrations. J Immunol Methods. 1992;153:125-131.
119. Cannon JJG, van der Meer JJW, Kwiatkowski DD, Endres SS, Lonnemann GG, Burke JJF, Dinarello CCA. Interleukin-1 beta in human plasma: Optimization of blood collection, plasma extraction, and radioimmunoassay methods. Lymphokine Res. 1988;7:457-467.
120. Thavasu PW, Longhurst S, Joel SP, Slevin ML, Balkwill FR. Measuring cytokine levels in blood: Importance of anticoagulants, processing, and storage conditions. J Immunol Methods. 1992;153:115-124.
121. Cannon JG, Nerad JL, Poutsiaka DD, Dinarello CA. Measuring circulating cytokines. J Appl Physiol. 1993;75:1897-1902.
122. Eriksson EE, Tengborn LL, Risberg BB. The effect of various anticoagulant/antiplatelet mixtures on determination of plasminogen activator inhibitor, platelet proteins and hemostasis parameters. Thromb Haemost. 1989;61:511-516.
123. Mire-Sluis AR, Gaines-Das R, Thorpe R. Immunoassays for detecting cytokines: What are they really measuring? J Immunol Methods. 1995;186:157-160.
124. Bustin SA. Absolute quantification of mRNA using real-time reverse transcription polymerase chain reaction assays. J Mol Endocrinol. 2000;25:169-193.
125. Hartel C, Bein G, Muller-Steinhardt M, Kluter H. Ex vivo induction of cytokine mRNA expression in human blood samples. J Immunol Methods. 2001;249:63-71.
126. Barth S, Kleinhappl B, Gutschi A, Jelovcan S, Marth E. In vitro cytokine mRNA expression in normal human peripheral blood mononuclear cells. Inflamm Res. 2000 49:266-274.
127. Schneider DJ, Tracy PB, Mann KG, Sobel BE. Differential effects of anticoagulants on the activation of platelets ex vivo. Circulation. 1997;96:2877-2883.
128. Aukrust P, Lien E, Kristoffersen AK, Muller F, Haug CJ, Espevik T, Frøland SS. Persistent activation of the tumor necrosis factor system in a subgroup of patients with common variable immunodeficiency--possible immunologic and clinical consequences. Blood. 1996;87:674-681.
129. Gerszten RE, Garcia-Zepeda EA, Lim YC, Yoshida M, Ding HA, Gimbrone MA, Luster

AD, Luscinskas FW, Rosenzweig A. MCP-1 and IL-8 trigger firm adhesion of monocytes to vascular endothelium under flow conditions. Nature. 1999;398:718-723.

130. Walz A, Baggiolini M. Generation of the neutrophil-activating peptide NAP-2 from platelet basic protein or connective tissue-activating peptide III through monocyte proteases. J Exp Med. 1990;171:449-454.

131. Pitsilos S, Hunt J, Mohler ER, Prabhakar AM, Poncz M, Dawicki J, Khalapyan TZ, Wolfe ML, Fairman R, Mitchell M, Carpenter J, Golden MA, Cines DB, Sachais BS. Platelet factor 4 localization in carotid atherosclerotic plaques: Correlation with clinical parameters. Thromb Haemost. 2003;90:1112-1120.

132. Traves SL, Smith SJ, Barnes PJ, Donnelly LE. Specific CXC but not CC chemokines cause elevated monocyte migration in COPD: A role for CXCR2. J Leukoc Biol. 2004;76:441-450.

133. Brandt E, Petersen F, Ludwig A, Ehlert J, Bock L, Flad H. The beta-thromboglobulins and platelet factor 4: Blood platelet-derived CXC chemokines with divergent roles in early neutrophil regulation. J Leukoc Biol. 2000;67:471-478.

134. Bazan JF, Bacon KB, Hardiman G, Wang W, Soo K, Rossi D, Greaves DR, Zlotnik A, Schall TJ. A new class of membrane-bound chemokine with a CX3C motif. Nature. 1997;385:640-644.

135. Matloubian M, David A, Engel S, Ryan JE, Cyster JG. A transmembrane CXC chemokine is a ligand for HIV-coreceptor Bonzo. Nat Immunol. 2000;1:298-304.

136. Shimaoka T, Kume N, Minami M, Hayashida K, Kataoka H, Kita T, Yonehara S. Molecular cloning of a novel scavenger receptor for oxidized low density lipoprotein, SR-PSOX, on macrophages. J Biol Chem. 2000;275:40663-40666.

137. Minami M, Kume N, Shimaoka T, Kataoka H, Hayashida K, Akiyama Y, Nagata I, Ando K, Nobuyoshi M, Hanyuu M, Komeda M, Yonehara S, Kita T. Expression of SR-PSOX, a novel cell-surface scavenger receptor for phosphatidylserine and oxidized LDL in human atherosclerotic lesions. Arterioscler Thromb Vasc Biol. 2001;21:1796-1800.

138. Wuttge DM, Zhou X, Sheikine Y, Wagsater D, Stemme V, Hedin U, Stemme S, Hansson GK, Sirsjo A. CXCL16/SR-PSOX is an interferon-{gamma}-regulated chemokine and scavenger receptor expressed in atherosclerotic lesions. Arterioscler Thromb Vasc Biol. 2004;24:750-755.

139. Hofnagel O, Luechtenborg B, Plenz G, Robenek H, Kume N. Expression of the novel scavenger receptor SR-PSOX in cultured aortic smooth muscle cells and umbilical endothelial cells. Arterioscler Thromb Vasc Biol. 2002;22:710-711.

140. Abel S, Hundhausen C, Mentlein R, Schulte A, Berkhout TA, Broadway N, Hartmann D, Sedlacek R, Dietrich S, Muetze B, Schuster B, Kallen KJ, Saftig P, Rose-John S, Ludwig A. The transmembrane CXC-chemokine ligand 16 is induced by IFN-{gamma} and TNF-{alpha} and shed by the activity of the disintegrin-like metalloproteinase ADAM10. J Immunol. 2004;172:6362-6372.

141. Shimaoka T, Nakayama T, Fukumoto N, Kume N, Takahashi S, Yamaguchi J, Minami M, Hayashida K, Kita T, Ohsumi J, Yoshie O, Yonehara S. Cell surface-anchored SR-PSOX/CXC chemokine ligand 16 mediates firm adhesion of CXC chemokine receptor 6-expressing cells. J Leukoc Biol. 2004;75:267-274.

142. Kageyama Y, Torikai E, Nagano A. Anti-tumor necrosis factor-alpha antibody treatment reduces serum CXCL16 levels in patients with rheumatoid arthritis. Rheumatol Int. 2006; [Epub ahead of print].

143. le Blanc LMP, van Lieshout AWT, Adema GJ, van Riel PLCM, Verbeek MM, Radstake TRDJ. CXCL16 is elevated in the cerebrospinal fluid versus serum and in inflammatory conditions with suspected and proved central nervous system involvement. Neuroscience Letters. 2006;397:145-148.
144. Ruth JH, Haas CS, Park CC, Amin MA, Martinez RJ, Haines GK, Shahrara S, Campbell PL, Koch AE. CXCL16-mediated cell recruitment to rheumatoid arthritis synovial tissue and murine lymph nodes is dependent upon the MAPK pathway. Arthritis Rheum. 2006;54:765-778.
145. Sheikine Y, Bang CS, Nilsson L, Samnegard A, Hamsten A, Jonasson L, Eriksson P, Sirsjo A. Decreased plasma CXCL16/SR-PSOX concentration is associated with coronary artery disease. Atherosclerosis. 2006;188:462-466.
146. Lehrke M, Millington SC, Lefterova M, Cumaranatunge RG, Szapary P, Wilensky R, Rader DJ, Lazar MA, Reilly MP. CXCL16 is a marker of inflammation, atherosclerosis, and acute coronary syndromes in humans. J Am Coll Cardiol. 2007;49:442-449.
147. Zhuge X, Murayama T, Arai H, Yamauchi R, Tanaka M, Shimaoka T, Yonehara S, Kume N, Yokode M, Kita T. CXCL16 is a novel angiogenic factor for human umbilical vein endothelial cells. Biochem Biophys Res Commun. 2005;331:1295-1300.
148. Lee SJ, Namkoong S, Kim YM, Kim CK, Lee H, Ha KS, Chung HT, Kwon YG, Kim YM. Fractalkine stimulates angiogenesis by activating the Raf-1/MEK/ERK- and PI3K/Akt/eNOS-dependent signal pathways. Am J Physiol Heart Circ Physiol. 2006;291:H2836-2846.
149. Gursel M, Gursel I, Mostowski HS, Klinman DM. CXCL16 influences the nature and specificity of CpG-induced immune activation. J Immunol. 2006;177:1575-1580.
150. Chandrasekar B, Bysani S, Mummidi S. CXCL16 signals via Gi, phosphatidylinositol 3-kinase, akt, I{kappa}B kinase, and nuclear factor-{kappa}B and induces cell-cell adhesion and aortic smooth muscle cell proliferation. J Biol Chem. 2004;279:3188-3196.
151. Aslanian AM, Charo IF. Targeted disruption of the scavenger receptor and chemokine CXCL16 accelerates atherosclerosis. Circulation. 2006;114:583-590.
152. Geissmann F, Cameron T, Sidobre S, Manlongat N, Kronenberg M, Briskin MJ, Dustin ML, Littman DR. Intravascular immune surveillance by CXCR6+ NKT cells patrolling liver sinusoids. PLoS Biol. 2005;3:e113.
153. Hase K, Murakami T, Takatsu H, Shimaoka T, Iimura M, Hamura K, Kawano K, Ohshima S, Chihara R, Itoh K, Yonehara S, Ohno H. The membrane-bound chemokine CXCL16 expressed on follicle-associated epithelium and M cells mediates lympho-epithelial interaction in GALT. J Immunol. 2006;176:43-51.
154. Garcia GE, Truong LD, Li P, Zhang P, Johnson RJ, Wilson CB, Feng L. Inhibition of CXCL16 attenuates inflammatory and progressive phases of anti-glomerular basement membrane antibody-associated glomerulonephritis. Am J Pathol. 2007;170:1485-1496.
155. Gough PJ, Garton KJ, Wille PT, Rychlewski M, Dempsey PJ, Raines EW. A disintegrin and metalloproteinase 10-mediated cleavage and shedding regulates the cell surface expression of CXC chemokine ligand 16. J Immunol. 2004;172:3678-3685.
156. Kiener PA, Davis PM, Murray JL, Youssef S, Rankin BM, Kowala M. Stimulation of inflammatory responses in vitro and in vivo by lipophilic HMG-CoA reductase inhibitors. Int Immunopharmacol. 2001;1:105-118.
157. Yoshie O, Imai T, Nomiyama H. Novel lymphocyte-specific CC chemokines and their receptors. J Leukoc Biol. 1997;62:634-644.

158. Camacho SA, Kosco-Vilbois MH, Berek C. The dynamic structure of the germinal center. Immunology Today. 1998;19:511-514.
159. Maclennan ICM. Germinal centers. Annu Rev Immunol. 1994;12:117-139.
160. Sallusto F, Schaerli P, Loetscher P, Schaniel C, Lenig D, Mackay CR, Qin S, Lanzavecchia A. Rapid and coordinated switch in chemokine receptor expression during dendritic cell maturation. Eur J Immunol. 1998;28:2760-2769.
161. Cyster JG. Chemokines and cell migration in secondary lymphoid organs. Science. 1999;286:2098-2102.
162. Cyster JG. Lymphoid organ development and cell migration. Immunol Rev. 2003;195:5-14.
163. Flanagan K, Moroziewicz D, Kwak H, Horig H, Kaufman HL. The lymphoid chemokine CCL21 costimulates naive T cell expansion and Th1 polarization of non-regulatory CD4+ T cells. Cell Immunol. 2004;231:75-84.
164. Marsland BJ, Battig P, Bauer M, Ruedl C, Lassing U, Beerli RR, Dietmeier K, Ivanova L, Pfister T, Vogt L, Nakano H, Nembrini C, Saudan P, Kopf M, Bachmann MF. CCL19 and CCL21 induce a potent proinflammatory differentiation program in licensed dendritic cells. Immunity. 2005;22:493-505.
165. Burman A, Haworth O, Hardie DL, Amft EN, Siewert C, Jackson DG, Salmon M, Buckley CD. A chemokine-dependent stromal induction mechanism for aberrant lymphocyte accumulation and compromised lymphatic return in rheumatoid arthritis. J Immunol. 2005;174:1693-1700.
166. Middel P, Raddatz D, Gunawan B, Haller F, Radzun HJ. Increased number of mature dendritic cells in Crohn's disease: Evidence for a chemokine mediated retention mechanism. Gut. 2006;55:220-227.
167. Holm AM, Sivertsen EA, Tunheim SH, Haug T, Bjerkeli V, Yndestad A, Aukrust P, Frøland SS. Gene expression analysis of peripheral T cells in a subgroup of common variable immunodeficiency shows predominance of CCR7 effector-memory T cells. Clin Exp Immunol. 2004;138:278-289.
168. Gafa V, Lande R, Gagliardi MC, Severa M, Giacomini E, Remoli ME, Nisini R, Ramoni C, Di Francesco P, Aldebert D, Grillot R, Coccia EM. Human dendritic cells following aspergillus fumigatus infection express the CCR7 receptor and a differential pattern of interleukin-12 (IL-12), IL-23, and IL-27 cytokines, which lead to a Th1 response. Infect Immun. 2006;74:1480-1489.
169. Chierchia S, Muiesan L, Davies A, Balasubramian V, Gerosa S, Raftery EB. Role of the sympathetic nervous system in the pathogenesis of chronic stable angina. Implications for the mechanism of action of beta-blockers. Circulation. 1990;82:71-81.
170. Erbel C, Sato K, Meyer FB, Kopecky SL, Frye RL, Goronzy JJ, Weyand CM. Functional profile of activated dendritic cells in unstable atherosclerotic plaque. Basic Res Cardiol. 2007;102:123-132.
171. Trogan E, Feig JE, Dogan S, Rothblat GH, Angeli V, Tacke F, Randolph GJ, Fisher EA. Gene expression changes in foam cells and the role of chemokine receptor CCR7 during atherosclerosis regression in ApoE-deficient mice. Proc Natl Acad Sci U S A. 2006;103:3781-3786.
172. Lefer AM, Scalia R, Lefer DJ. Vascular effects of HMG CoA-reductase inhibitors (statins) unrelated to cholesterol lowering: new concepts for cardiovascular disease. Cardiovasc Res. 2001;49:281-287.

173. Schonbeck U, Libby P. Inflammation, immunity, and HMG-CoA reductase inhibitors: statins as antiinflammatory agents? Circulation. 2004;109:II-18-26.
174. Bromley SK, Thomas SY, Luster AD. Chemokine receptor CCR7 guides T cell exit from peripheral tissues and entry into afferent lymphatics. Nat Immunol. 2005;6:895-901.
175. Debes GF, Arnold C, Young AJ, Krautwald S, Lipp M, Hay JB, Butcher EC. Chemokine receptor CCR7 required for T lymphocyte exit from peripheral tissues. Nat Immunol. 2005;69:889-894.
176. Yilmaz A, Reiss C, Weng A, Cicha I, Stumpf C, Steinkasserer A, Daniel WG, Garlichs CD. Differential effects of statins on relevant functions of human monocyte-derived dendritic cells. J Leukoc Biol. 2006;79:529-538.
177. Pedersen TR, Faergeman O, Kastelein JJP, Olsson AG, Tikkanen MJ, Holme I, Larsen ML, Bendiksen FS, Lindahl C, Szarek M, Tsai J. High-dose atorvastatin vs usual-dose simvastatin for secondary prevention after myocardial infarction: The IDEAL study: A randomized controlled trial. JAMA. 2005;294:2437-2445.
178. Ahmad R, Menezes J, Knafo L, Ahmad A. Activated human platelets express Fas-L and induce apoptosis in Fas-positive tumor cells. J Leukoc Biol. 2001;69:123-128.
179. Cohavy O, Zhou J, Ware CF, Targan SR. LIGHT is constitutively expressed on T and NK cells in the human gut and can be induced by CD2-mediated signaling. J Immunol. 2005;174:646-653.
180. Kim WJ, Kang YJ, Koh EM, Ahn KS, Cha HS, Lee WH. LIGHT is involved in the pathogenesis of rheumatoid arthritis by inducing the expression of pro-inflammatory cytokines and MMP-9 in macrophages. Immunology. 2005;114:272-279.
181. Schneider K, Potter KG, Ware CF. Lymphotoxin and LIGHT signaling pathways and target genes. Immunol Rev. 2004;202:49-66.
182. Shaikh RB, Santee S, Granger SW, Butrovich K, Cheung T, Kronenberg M, Cheroutre H, Ware CF. Constitutive expression of LIGHT on T cells leads to lymphocyte activation, inflammation, and tissue destruction. J Immunol. 2001;167:6330-6337.
183. Wang J, Lo JC, Foster A, Yu P, Chen HM, Wang Y, Tamada K, Chen L, Fu YX. The regulation of T cell homeostasis and autoimmunity by T cell-derived LIGHT. J Clin Invest. 2001;108:1771-1780.
184. Lee WH, Kim SH, Lee Y, Lee BB, Kwon B, Song H, Kwon BS, Park JE. Tumor necrosis factor receptor superfamily 14 is involved in atherogenesis by inducing proinflammatory cytokines and matrix metalloproteinases. Arterioscler Thromb Vasc Biol. 2001;21:2004-2010.
185. Scholz H, Sandberg W, Damås JK, Smith C, Andreassen AK, Gullestad L, Frøland SS, Yndestad A, Aukrust P, Halvorsen B. Enhanced plasma levels of LIGHT in unstable angina: possible pathogenic role in foam cell formation and thrombosis. Circulation. 2005;112:2121-2129.
186. Hehlgans T, Mannel DN. Recombinant, soluble LIGHT (HVEM Ligand) induces increased IL-8 secretion and growth arrest in A375 melanoma cells. J Interferon Cytokine Res. 2001;21:333-338.
187. Anand S, Wang P, Yoshimura K, Choi IH, Hilliard A, Chen YH, Wang CR, Schulick R, Flies AS, Flies DB, Zhu G, Xu Y, Pardoll DM, Chen L, Tamada K. Essential role of TNF family molecule LIGHT as a cytokine in the pathogenesis of hepatitis. J Clin Invest. 2006;116:1045-1051.
188. Heo SK, Ju SA, Lee SC, Park SM, Choe SY, Kwon B, Kwon BS, Kim BS. LIGHT

enhances the bactericidal activity of human monocytes and neutrophils via HVEM. J Leukoc Biol. 2006;79:330-338.

189. Damås JK, Otterdal K, Yndestad A, Aass H, Solum NO, Frøland SS, Simonsen S, Aukrust P, Andreassen AK. Soluble CD40 ligand in pulmonary arterial hypertension: Possible pathogenic role of the interaction between platelets and endothelial cells. Circulation. 2004;110:999-1005.

190. Wei CY, Chou YH, Ho FM, Hsieh SL, Lin WW. Signaling pathways of LIGHT induced macrophage migration and vascular smooth muscle cell proliferation. J Cell Physiol. 2006;209:735-743.

191. Lo JC, Wang Y, Tumanov AV, Bamji M, Yao Z, Reardon CA, Getz GS, Fu YX. Lymphotoxin beta receptor-dependent control of lipid homeostasis. Science. 2007;316:285-288.

192. Yndestad A, Ueland T, Øie E, Florholmen G, Halvorsen B, Attramadal H, Simonsen S, Frøland SS, Gullestad L, Christensen G, Damås JK, Aukrust P. Elevated levels of activin A in heart failure. Potential role in myocardial remodeling. Circulation. 2004;109:1379-1385.

193. Matsuse T, Ikegami A, Ohga E, Hosoi T, Oka T, Kida K, Fukayama M, Inoue S, Nagase T, Ouchi Y, Fukuchi Y. Expression of immunoreactive activin A protein in remodeling lesions associated with interstitial pulmonary fibrosis. Am J Pathol. 1996;148:707-713.

194. Cho SH, Yao Z, Wang SW, Alban RF, Barbers RG, French SW, Oh CK. Regulation of activin A expression in mast cells and asthma: its effect on the proliferation of human airway smooth muscle cells. J Immunol. 2003;170:4045-4052.

195. Yu EW, Dolter KE, Shao LE, Yu J. Suppression of IL-6 biological activities by activin A and implications for inflammatory arthropathies. Clin Exp Immunol. 1998;112:126-132.

196. Jones KL, Brauman JN, Groome NP, de Kretser DM, Phillips DJ. Activin A release into the circulation is an early event in systemic inflammation and precedes the release of follistatin. Endocrinology. 2000;141:1905-1908.

197. Inoue S, Orimo A, Hosoi T, Matsuse T, Hashimoto M, Yamada R, Ouchi Y, Orimo H, Muramatsu M. Expression of follistatin, an activin-binding protein, in vascular smooth muscle cells and arteriosclerotic lesions. Arterioscler Thromb. 1993;13:1859-1864.

198. Inoue S, Orimo A, Hosoi T, Matsuse T, Kozaki K, Ouchi Y, Muramatsu M, Orimo H. Follistatin as an activin-binding protein expressed in arteriosclerotic lesions. Ann N Y Acad Sci. 1995;748:530-533.

199. Engelse MA, Lardenoye JHP, Neele JM, Grimbergen JM, de Vries MR, Lamfers MLM, Pannekoek H, Quax PHA, de Vries CJM. Adenoviral activin A expression prevents intimal hyperplasia in human and murine blood vessels by maintaining the contractile smooth muscle cell phenotype. Circ Res. 2002;90:1128-1134.

200. Engelse MA, Neele JM, van Achterberg TA, van Aken BE, van Schaik RH, Pannekoek H, de Vries CJ. Human activin-A is expressed in the atherosclerotic lesion and promotes the contractile phenotype of smooth muscle cells. Circ Res. 1999;85:931-939.

201. Biasucci LM, Vitelli A, Liuzzo G, Altamura S, Caligiuri G, Monaco C, Rebuzzi AG, Ciliberto G, Maseri A. Elevated levels of interleukin-6 in unstable angina. Circulation. 1996;94:874-877.

202. Yamashita H, Shimada K, Seki E, Mokuno H, Daida H. Concentrations of interleukins, interferon, and C-reactive protein in stable and unstable angina pectoris. Am J Cardiol. 2003;91:133-136.

203. Nusing RM, Barsig J. Induction of prostanoid, nitric oxide, and cytokine formation in rat

bone marrow derived macrophages by activin A. Br J Pharmacol. 1999;127:919-926.

204. Petraglia F, Sacerdote P, Cossarizza A, Angioni S, Genazzani AD, Franceschi C, Muscettola M, Grasso G. Inhibin and activin modulate human monocyte chemotaxis and human lymphocyte interferon-gamma production. J Clin Endocrinol Metab. 1991;72:496-502.

205. Keelan JA, Zou RL, Mitchell MD. Activin A exerts both pro- and anti-inflammatory effects on human term gestational tissues. Placenta. 2000;21:38-43.

206. Hedger MP, Phillips DJ, de Kretser DM. Divergent cell-specific effects of activin-A on thymocyte proliferation stimulated by phytohemagglutinin, and interleukin 1beta or interleukin 6 in vitro. Cytokine. 2000;12:595-602.

207. Brosh N, Sternberg D, Honigwachs-Sha'anani J, Lee BC, Shav-Tal Y, Tzehoval E, Shulman LM, Toledo J, Hacham Y, Carmi P, et al. The plasmacytoma growth inhibitor restrictin-P is an antagonist of interleukin 6 and interleukin 11. J Biol Chem. 1995;270:29594-29600.

208. Feinberg MW, Watanabe M, Lebedeva MA, Depina AS, Hanai J, Mammoto T, Frederick JP, Wang XF, Sukhatme VP, Jain MK. Transforming growth factor-{beta}1 inhibition of vascular smooth muscle cell activation is mediated via Smad3. J Biol Chem. 2004;279:16388-16393.